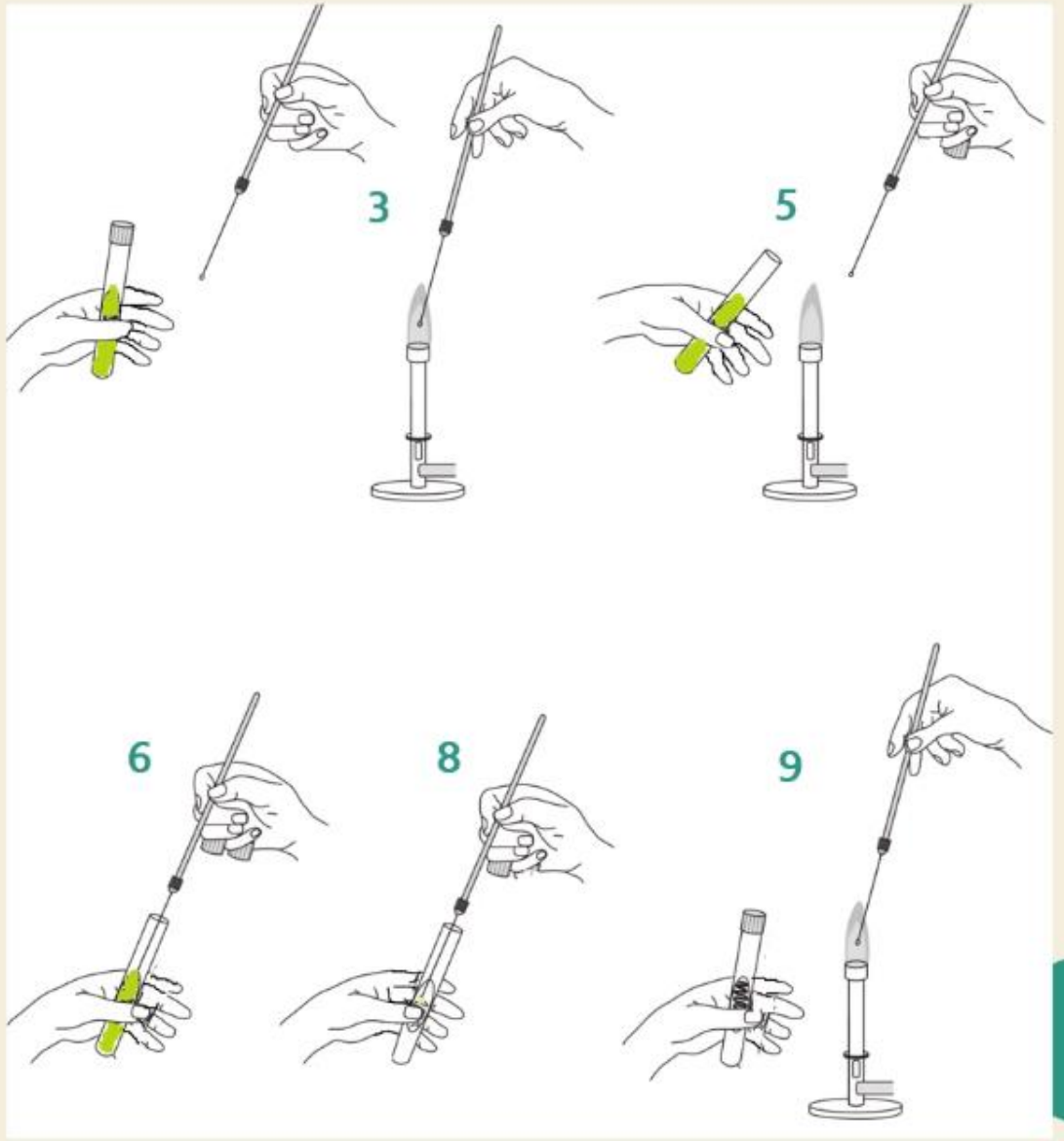
PRACTICAL 1

Broth transfer

1. Bunsen burner on white tile
2. Vortex culture
3. Flame ENTIRE inoculating loop till red hot
4. Wait 10 seconds for cooling
5. Flame mouth of test tube
6. Insert loop, remove loop-full of culture
7. Flame mouth of test tube and close
8. Flame mouth of sterile test tube, insert loop, flame mouth of test tube and re-cap
9. Flame inoculating loop till red hot and place on tile
10. Label and place product in CORRECT baskets for incubation

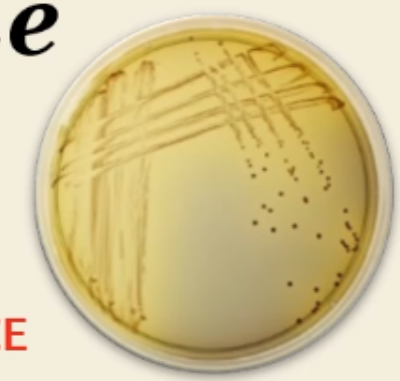


Pour plate technique

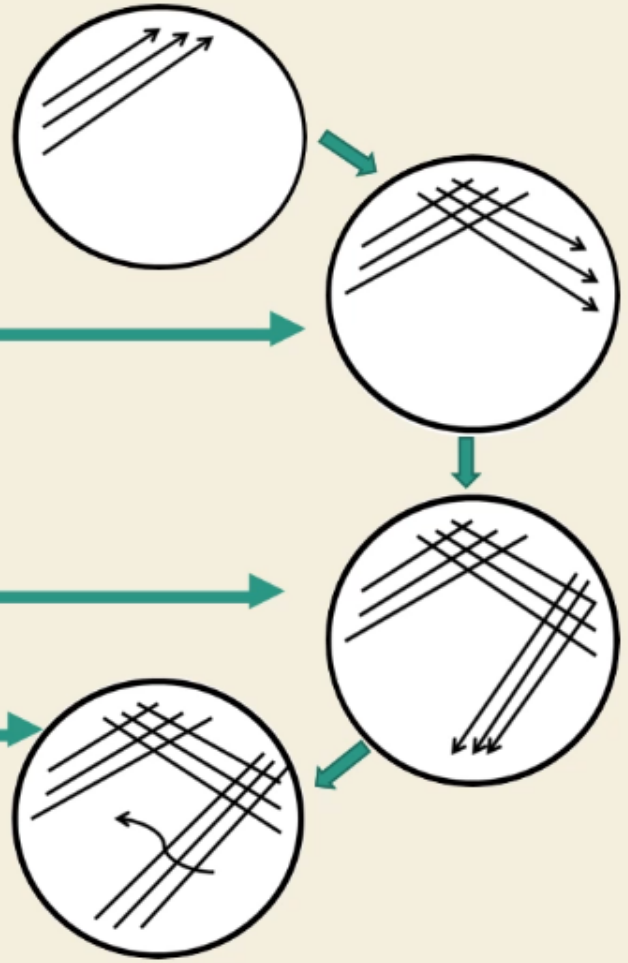
1. Label BOTTOM of petri-dish appropriately
2. Vortex culture
3. Pipette 0.1 ml culture into dish using the pre-set micro-pipettes
4. Let molten agar cool a bit
5. Pipette 20 ml agar (uniformly and without bubbles) into dish. DO NOT SHAKE dish
6. Allow agar to harden
7. INVERT and incubate in CORRECT baskets
8. Molten agar MUST be returned to water bath

Purpose : Count number of viable colonies in a sample.
Advantage : Can detect lower [ ] than spread plate

Streak plate technique



1. Label **BOTTOM** of petri-dish
2. Vortex culture, flame mouth of test tube
3. Flame loop, allow to cool and remove loop-full of culture **ONCE**
4. Streak culture in 3 parallel lines
5. **Flame loop**
6. Streak 3 parallel lines
7. **Flame loop**
8. Streak 3 parallel lines
9. **Flame loop**
10. Streak a "S" shape line
11. **Flame loop**
12. **INVERT** and incubate plate in **CORRECT** baskets



Purpose: Determine purity of culture . Get single colonies . 1 colony= pure

Important points to remember with the streak plate technique:

1. Parallel lines – should not cross
2. Do not dig into the agar
3. Flame loop between a set of lines streaked.
4. Make sure the loop is cool before you streak again

Spread plate technique

1) Label **BOTTOM** of petri-dish
2) Flame mouth of test tube of culture
3) Pipette 0.1 ml of culture onto agar
4) Spread culture over agar
5) **INVERT** and incubate

Purpose : observe uniform growth of culture and isolated colonies become easier to count when spread out. Can determine number of CFU.

Air sampling (demo)

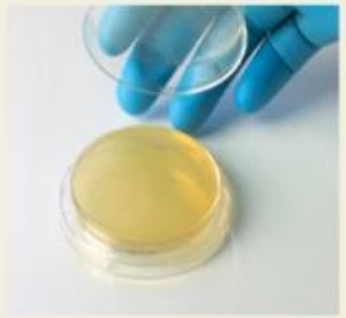
Types of air sampling

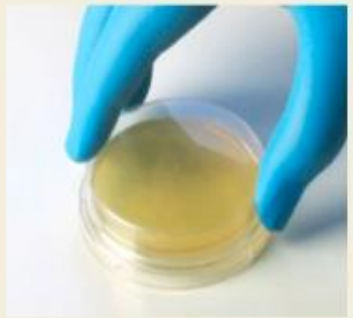
1. **Sedimentation**: passive air sampling using normal agar plates, also known as settle plates in this instance.
2. **Impaction**: this is how air samplers work (RCS and SAS), particles are accelerated out of the air and deposited onto the surface of the agar

Contact plates

Purpose of contact plate?

To evaluate the contamination on surfaces and determine the type of micro-organisms present by making the bacterial load on the surface visible.





Air sampling using impaction

1. Reuter centrifugal sampler (RCS)

- Running time: 4 minutes
- Volume of air : 280 L
- Size detection limit: 0.4 µm

2. Surface Air Sampler (SAS)

- Running time: 400 seconds
- Volume of air : 180 L
- Size detection limit : 2 µm

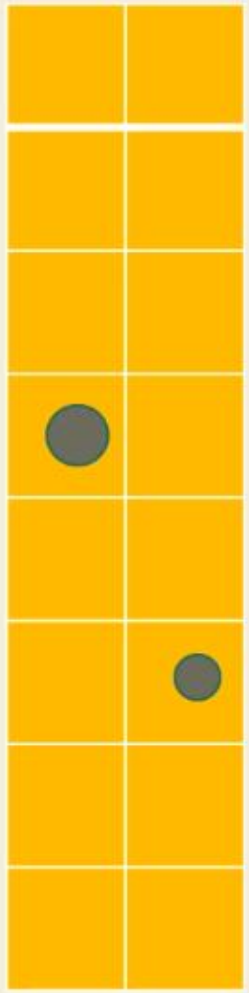
Results

- Viable Count – count of only live micro-organisms
- Total Count – count of dead and live microorganisms

Air Sampling Calculation

- CFU/L= (No. of colonies/ V air)
- **2 colonies**; RCS air sampler

- CFU/L (sec) = **2/(280/240)**
- RCS = **1.71** CFU/L per second



Air Sampling Calculation

- CFU/L= (No. of colonies/ V air)
- **26 colonies**; RCS air sampler

- CFU/L (sec) = **26/(280/240)**
- RCS = **22.29** CFU/L per second

Air sampling

SAS	RCS	Settle/Contact plates
Active	Active	Passive
Requires charging	Battery powered	Manual
Diffucult to operate	Easy to operate	Easy to operate
no autoclaving	Can be autoclaved	-
bulky transportation	Easier to transport	Easy to transport
Lower micro-organism recovery	Higher micro-organism recovery	lowest micro-organism recovery
no fungal spores <2µm	Low detection limit of 0.4µm	High detection limit
quantitative & qualitative	quantitative & qualitative	Only qualitative

PRACTICAL 2

1. GROWTH MEDIA

Medium	Pathogen	Example
Nutrient agar (NA) or broth (NB)	Bacteria	*S. aureus, P. aeruginosa*
Tryptone Soya agar (TSA) or broth (TSB)	Yeast, bacteria	*C. albicans, S. aureus*
Thioglycollate broth (TGB)	Anaerobes	*C. welchi, P. acnes*
Sebrauds dextrose broth (SDB) or agar (SDA)	Yeast and dermatophytes	*C. albicans, Trichophyton* spp., *Microsporum* ssp.
Meuller hinton broth supplemented with 5% sheep's blood (MHB) or agar (MHA)	Coagulase bacteria	*Streptococci* (*mutans, pyogenes*)
Rogosa broth (RB) or agar (RA)	Acid environment	*Lactobacillus*

RESULTS: TEMPERATURE

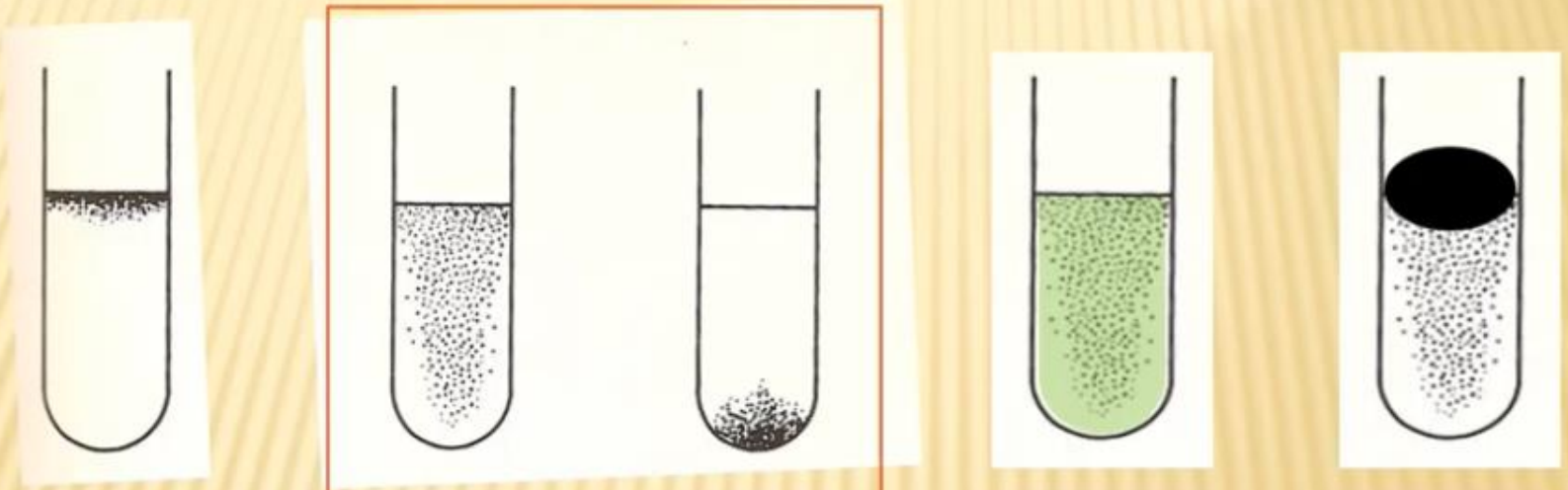
Micro-Organism	Temperature			
	4 ˚C	25 ˚C	37 ˚C	55 ˚C
E. coli	–	++	+++	–
B. stearothermophilus	–	–	++	+++
C. albicans	–	+++	++	–

INFLUENCE OF TEMPERATURE AND TIME

Organisms	Temperature	Incubation time
Facultative bacteria	37 ˚C	24 hrs
Yeast	25 ˚C	48 hrs
Dermatophytes	30 ˚C	7 days
Fastidious anaerobe	37 ˚C	2-7 days

3. GROWTH PATTERNS

Growth patterns of micro-organisms:



AEROBE:
Mycobacterium

FACULTATIVE
E. coli

OBLIGATE
P. acnes
Clostridium

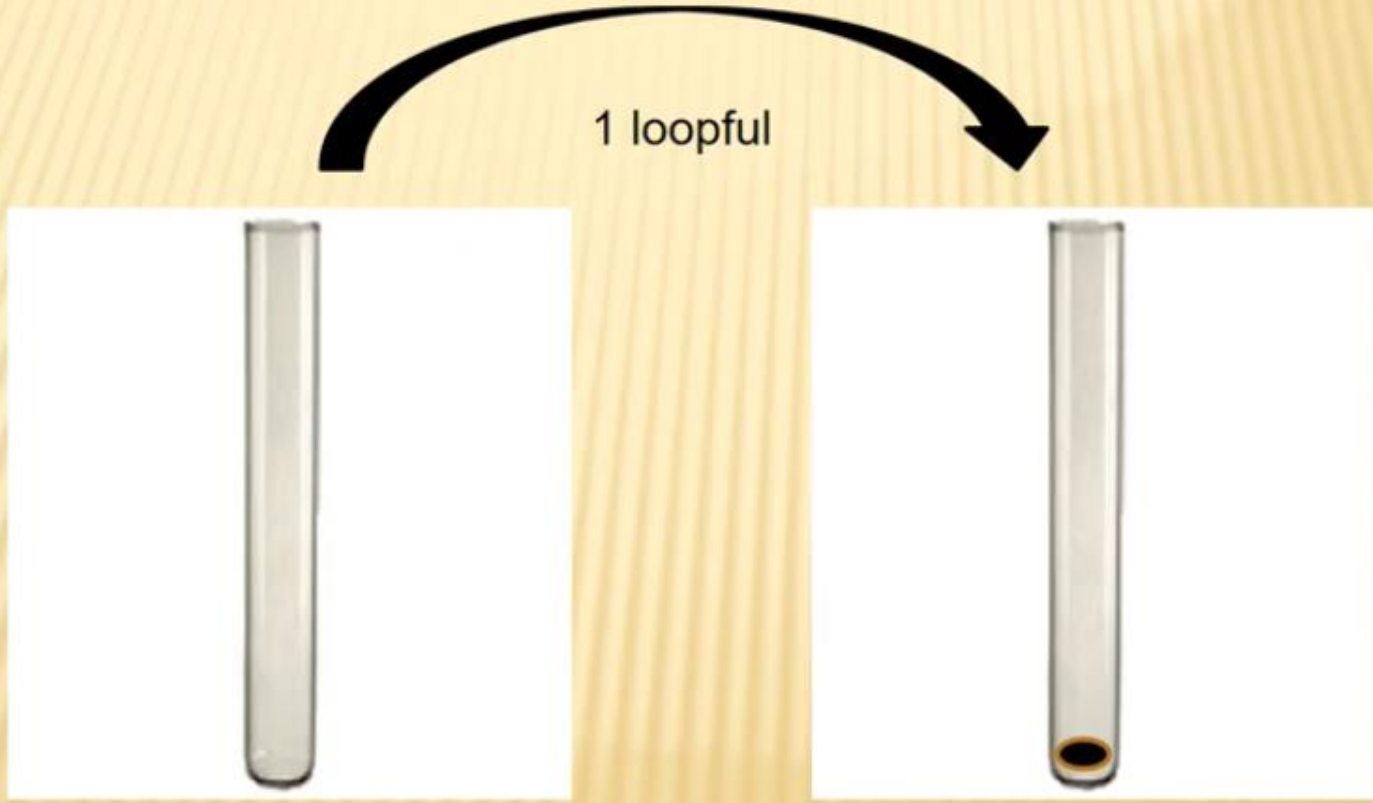
ANAEROBE

PIGMENT
P. aeruginosa
S. marcenses
C. violaceum

PELLICLE
B. cereus

4. OXYGEN REQUIREMENTS

- 1. Anaerobic media

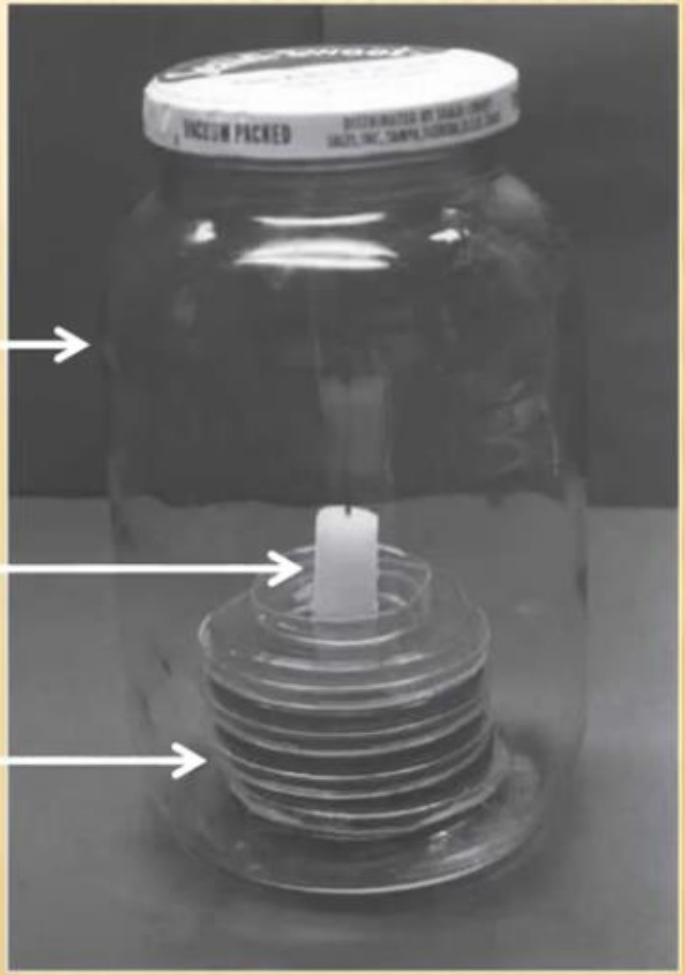


Clostridium welchii

Thioglycollate media

4. OXYGEN REQUIREMENTS

- 2. Anaerobic candle jar
- Anaerobes: Facultative



4. OXYGEN REQUIREMENTS

3. Anaerobic gas pack: Obligate and fastidious anaerobes

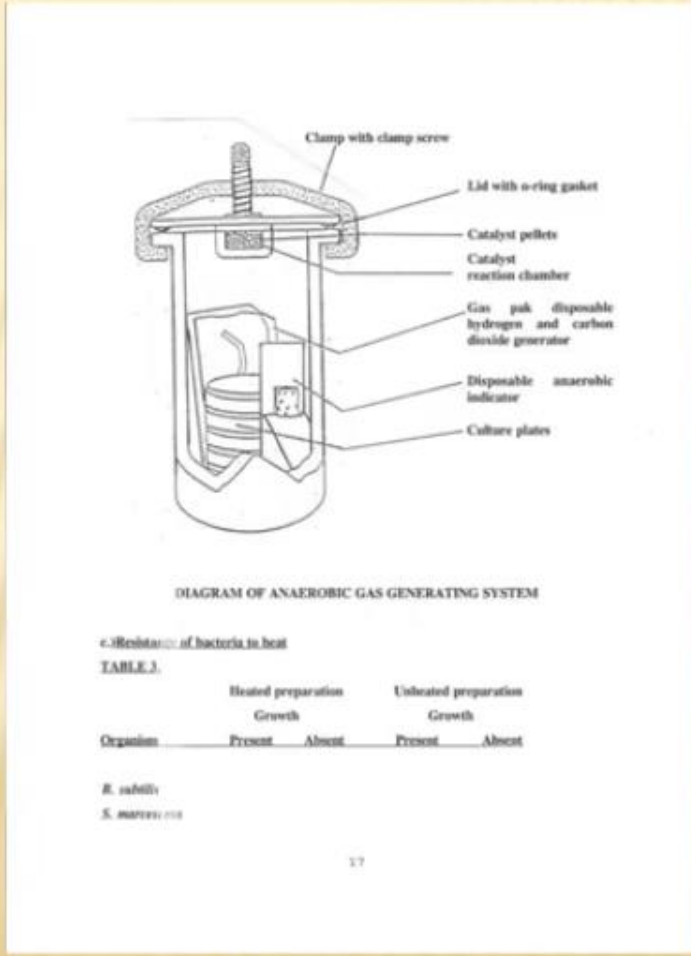


DIAGRAM OF ANAEROBIC GAS GENERATING SYSTEM

c.)Resistance of bacteria to heat

TABLE 3.

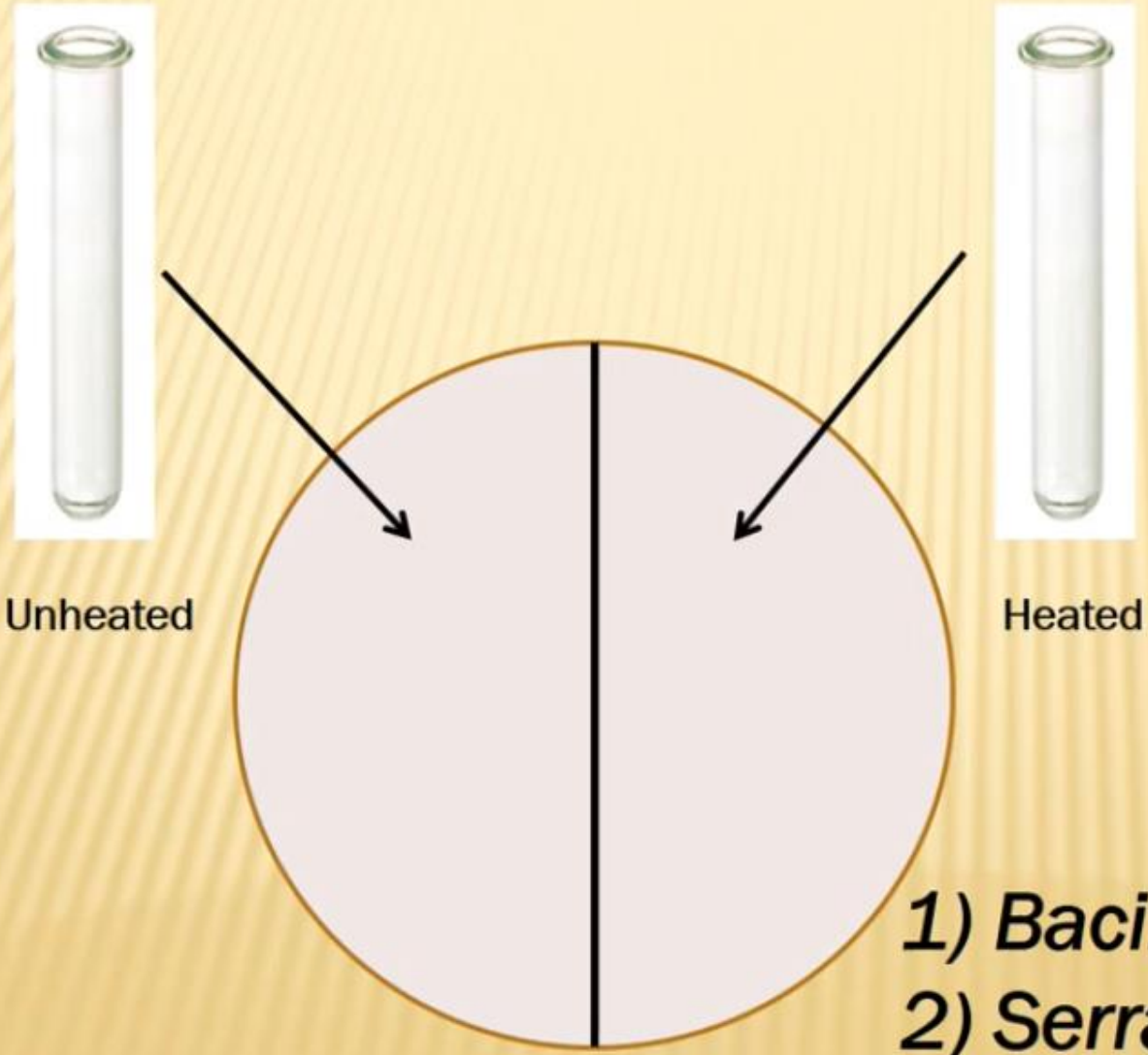
Organism	Heated preparation Growth Present	Heated preparation Growth Absent	Unheated preparation Growth Present	Unheated preparation Growth Absent
B. subtilis				
S. marcescens				

17

Gas Pack Method

- place 1 set of streaked plates inverted into jar
- Prepare generator by tearing off corner of envelope. Introduce 10ml sterile distilled water into gas pack.
- Open indicator envelope to expose indicator strip and place in jar. (must be visible from outside)
- Place lid and screw clamp in position on the jar
- Tighten screw clamp in position on jar
- Check indicator strip periodically for change from pink → white. If does not occur in 1-2hrs, replace generator
- Examine streak plate after incubation, draw appearance, comment

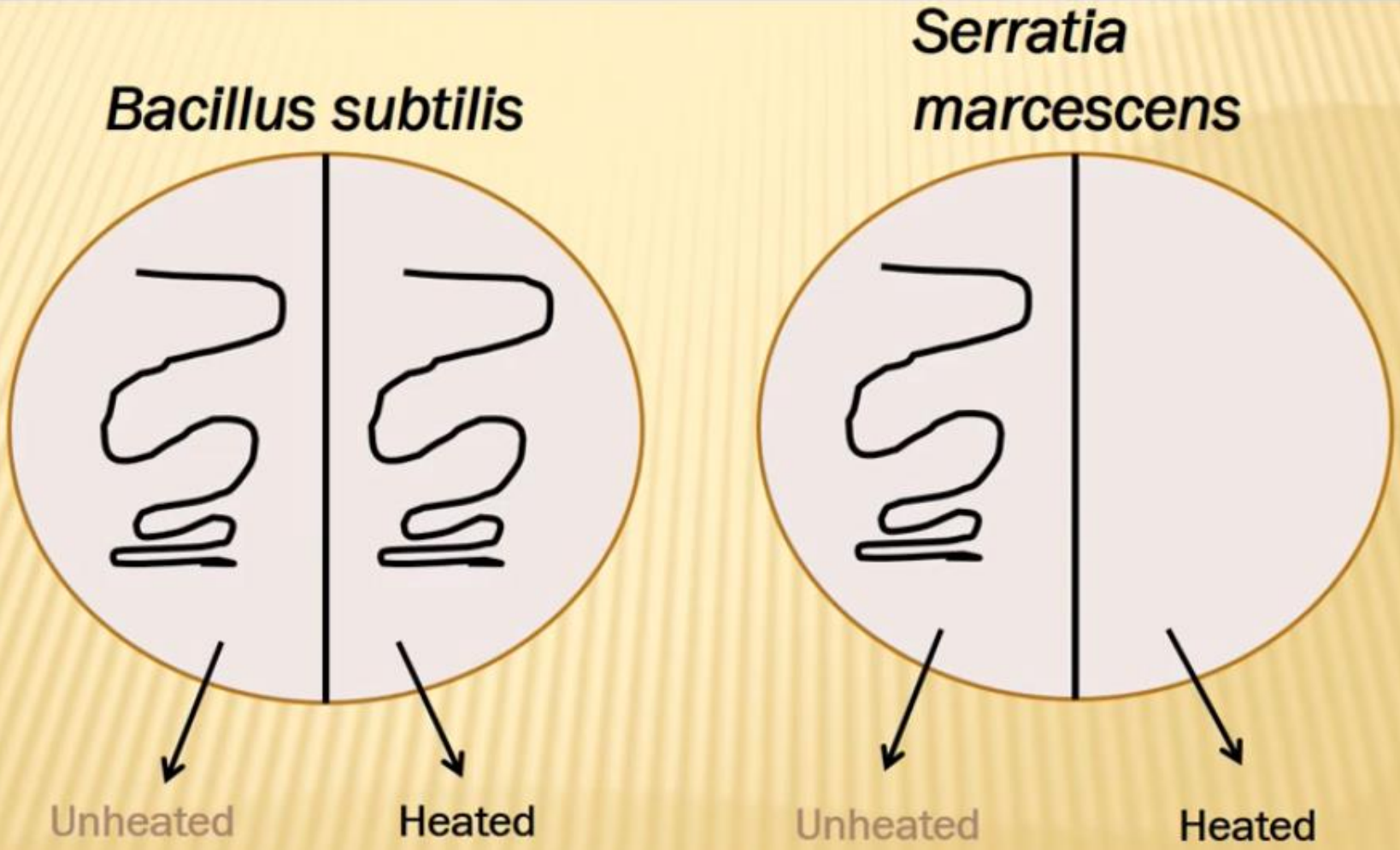
5. RESISTANCE TO HEAT



1) Bacillus subtilis

2) Serratia marcescens

5. RESISTANCE TO HEAT



6. SPORES VS BIOFILMS

Spores: dormant, reproductive cells, with thick-walls ,and is highly resistant in order to survive under unfavorable conditions; so that when conditions are again suitable, a new organisms grows . E.g dermatophytes, Baccilli.

Biofilm: A complex structure adhering to surfaces that are regularly in contact with moisture; consists of bacterial and fungal colonies. Secrete a protective coating in which they are encased

E.g *Strep. mutans*., *Candida*, *S. epidermidis*

http://www.biology-online.org/dictionary/Spore

TOTAL COUNT VS VIABLE COUNT

Total Counts (dead and alive)

Microscope

Spectrophotometer

Electronic

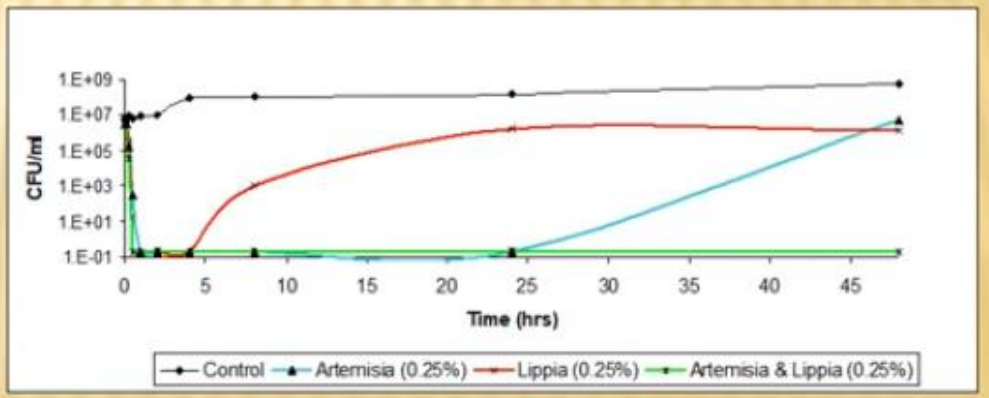
Viable Counts (alive only)

Pour plates

Membrane filtration

Spread plates

Time-kill



MICRO-ORGANISMS AVAILABLE FOR THIS PRACTICAL

- *Staphylococcus aureus*
- *Escherichia coli*
- *Candida albicans*
- *Pseudomonas aeruginosa*
- *Bacillus cereus*

	S. Aureus	E. Coli	C. albicans	P. aeruginosa	B. cereus
Susceptible antibiotics	Chloramphenicol Erythromycin Fusidic acid	Chloramphenicol Metronidazole Penicillin	Amphotericin B Nystatin Penicillin	Ciprofloxacin Gentamicin Penicillin	Chloramphenicol Erythromycin Gentamicin
Resistant Antibiotics	Tetracycline	Ciprofloxacin	Fusidic acid	Tetracyclines	Penicillin

TESTING METHODS

AGAR DIFFUSION

1. WELL PLATE METHOD
2. DISK DIFFUSION METHOD

Qualitative

TURBIDIMETRIC ASSAYS (DEMO)

3. MICRO-DILUTION METHOD
4. MACRO-DILUTION METHOD

Turbid broth – qualitative and quantitative

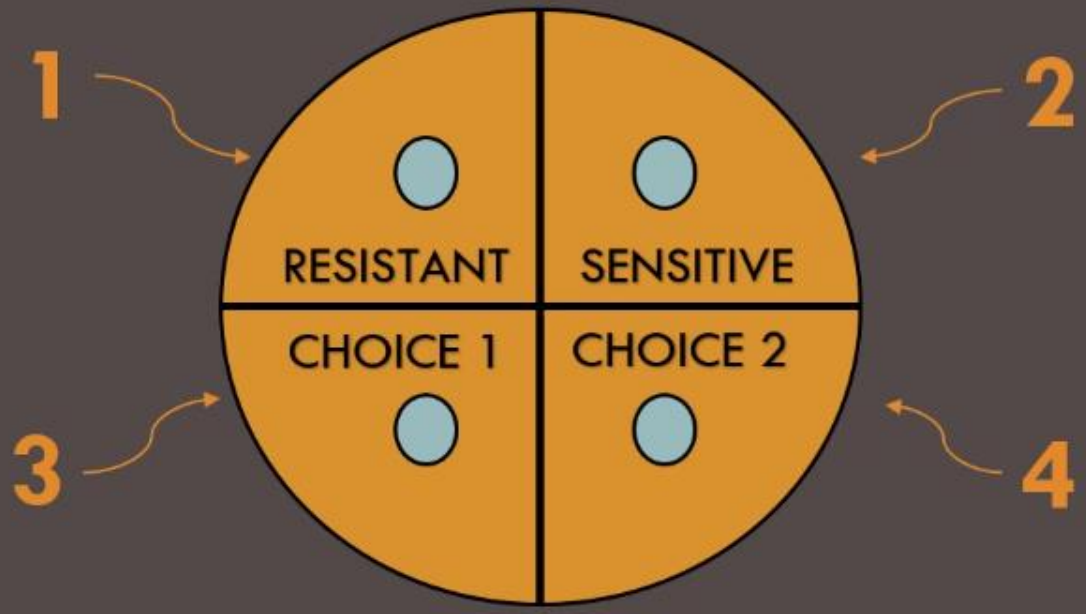
1. WELL PLATE METHOD: STEPS

1. **POUR PLATE:** 0.1ml of culture into empty sterile petri dish then add 20ml sterile molten agar.
2. Once solidified, make holes in the agar using back of sterile pasteur pipette.
3. Remove agar plugs and discard appropriately. Standard solutions of 4 different antimicrobials are then pipetted into the wells.
4. Cover plates and allow to **pre-diffuse for 1 hour.**
5. Incubate appropriately.
6. No need to invert well plates

ANTIMICROBIALS

Test	Advantages	Disadvantages
Wells	Can alter [] of antimicrobial Variables can be controlled	Lengthy Accuracy reduced- more subject to human error
Disc	Accurate –less subject to human error Quick	Antimicrobial in fixed [] Dependent on type of organism

Choose 4 different antimicrobial solutions



1. WELL PLATE METHOD

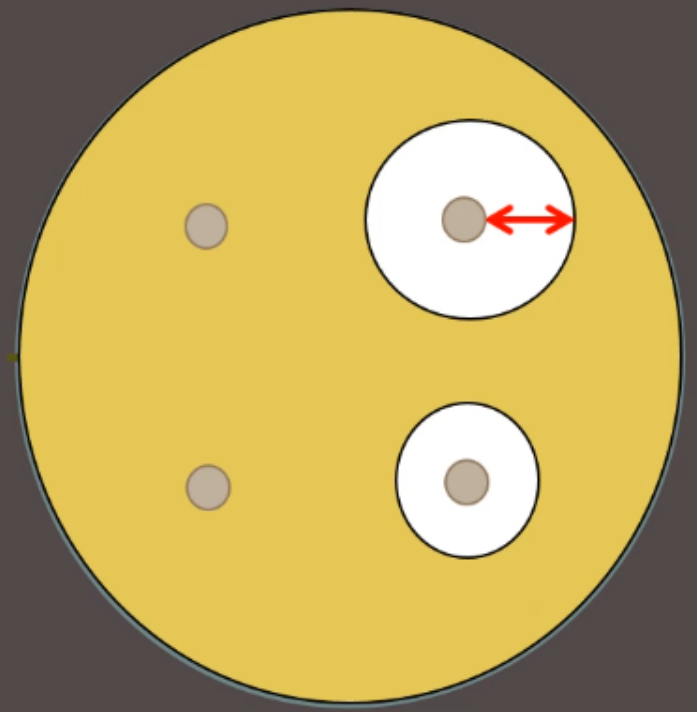
- Cavities or **wells** which are created in an **inoculated** agar medium
- **Antimicrobial solutions** are placed in these wells.
- Solution diffuses through the surrounding agar to create a **zone of inhibition**.
- Radius of inhibition zone reflects the concentration of the antimicrobial and the intrinsic sensitivity of the organism.

RESULTS

After incubation:

Measure zones of inhibition around each well.

The zone of inhibition is measured with a pair of calipers/ruler (mm).



2. DISK DIFFUSION METHOD

- Commercial antibiotic **paper disks** are placed on the surface of the **pour plate**.
- **NO WELLS**
- A **zone of inhibition** forms on the pour plate around the paper disk.
- Radius of inhibition zone reflects the concentration of the antimicrobial and the intrinsic sensitivity of the organism.

2. DISK DIFFUSION METHOD: STEPS

1. POUR PLATE: 0.1 ml of culture pipetted into center a sterile petri dish.
2. Pipette 20 ml of molten agar onto culture.
3. Once solidified, place antimicrobial disks onto surface of agar, using the sterile needle.
4. **NB**: Do not use the same needle for different antibiotic disks – CROSS CONTAMINATION
5. Make sure disks are placed equidistant from each other.

1. Use **same** test organisms as with well plate method.
2. Incubate appropriately.
3. Results: Zone of inhibition – measure radius.

2. DISK DIFFUSION METHOD: STEPS

1. Work in groups of two – work with your bench partner.
2. Each student to bring a commercial product that has antimicrobial properties.
3. The concentration of the active ingredient MUST be known.
4. Soak blank paper disks in sterile petri dishes containing each of the commercial products.

3. DISK DIFFUSION ON COMMERCIAL PRODUCT

5. Each group of two students must do three plates.
6. Each plate must have a different micro-organism, either S. aureus, E. Coli and C. albicans
7. Prepare spread plates for this exercise – NOT pour plates.

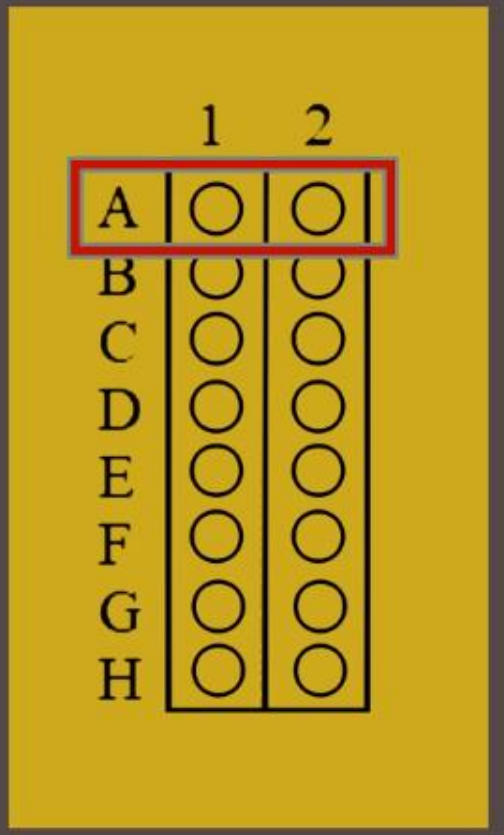
3. DISK DIFFUSION ON COMMERCIAL PRODUCT

1. Three SPREAD PLATES – not pour plates
2. SPREAD PLATE: 0.1 ml of culture onto a sterile agar plate. Use spreader to spread it out.
3. REMEMBER to spread the culture evenly with your spread plate, getting as close as possible to the edge without touching the edge.

3. DISK DIFFUSION ON COMMERCIAL PRODUCT – METHOD

CALCULATING MIC

100 µl of clean broth in the well



100 µl of the stock sample, with a concentration of 32 mg/ml is added to Row A

$$C_1V_1 = C_2V_2$$
$$(32\ mg/ml)(100\ \mu l) = C_2\ (200\ \mu l)$$
$$C_2 = 16\ mg/ml$$

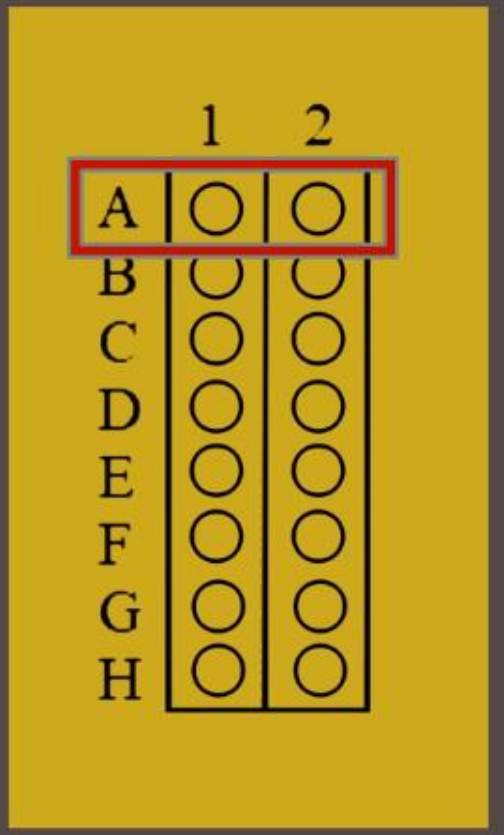
OR

$$C = \frac{n}{V}$$

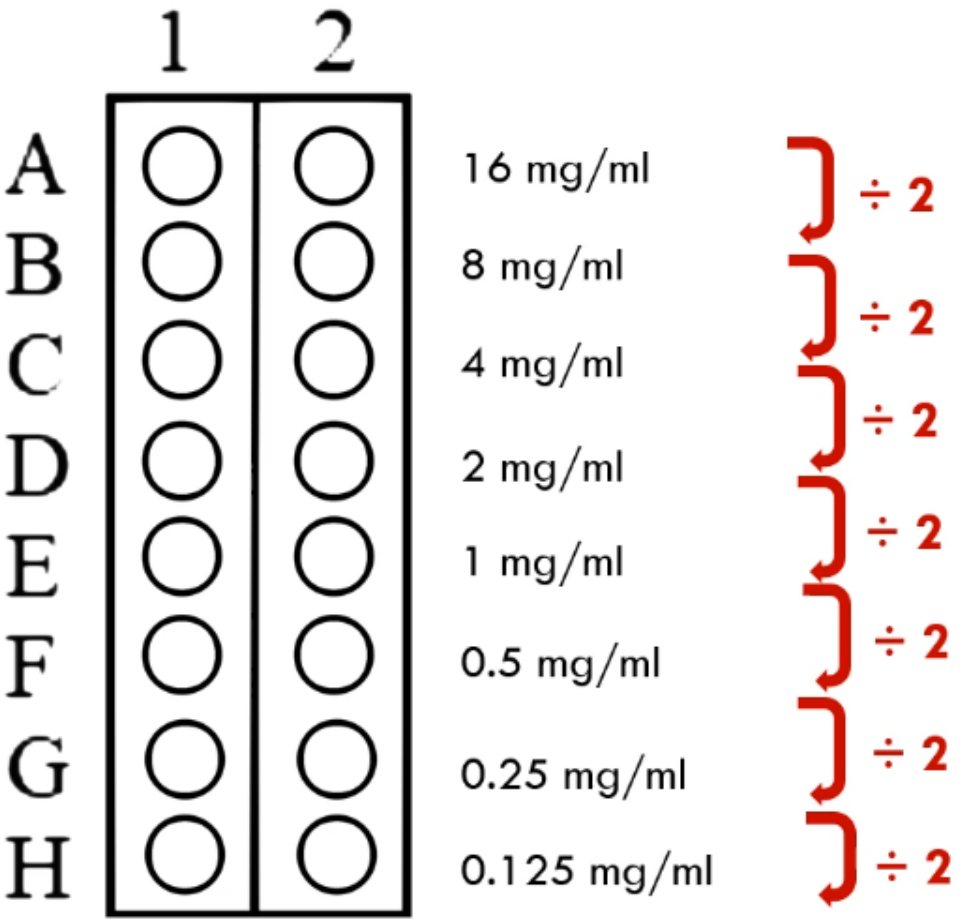
÷ 2 ↓ ↑ X 2

CALCULATING MIC

Serially diluted wells and discarded 100 µl at the end, therefore each well has 100 µl. Row A's wells have a concentration of 16 mg/ml now.



CALCULATING MIC

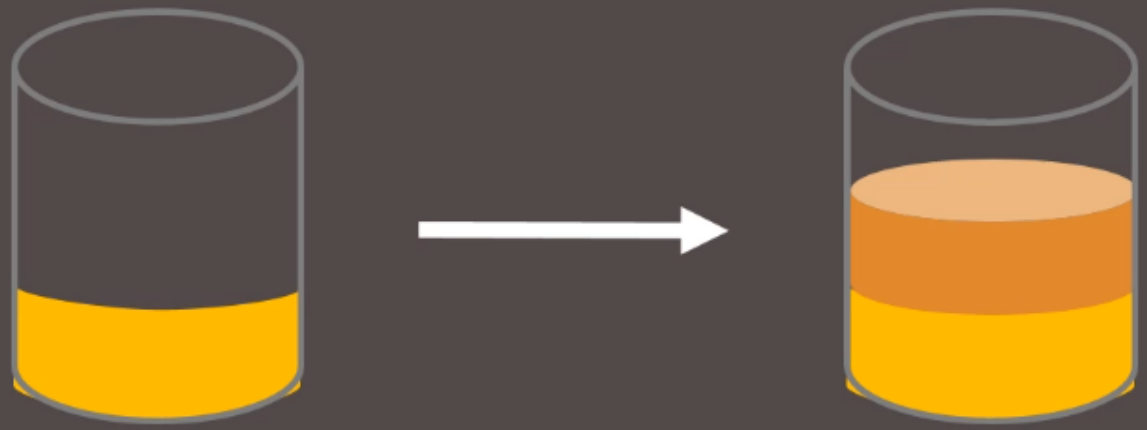


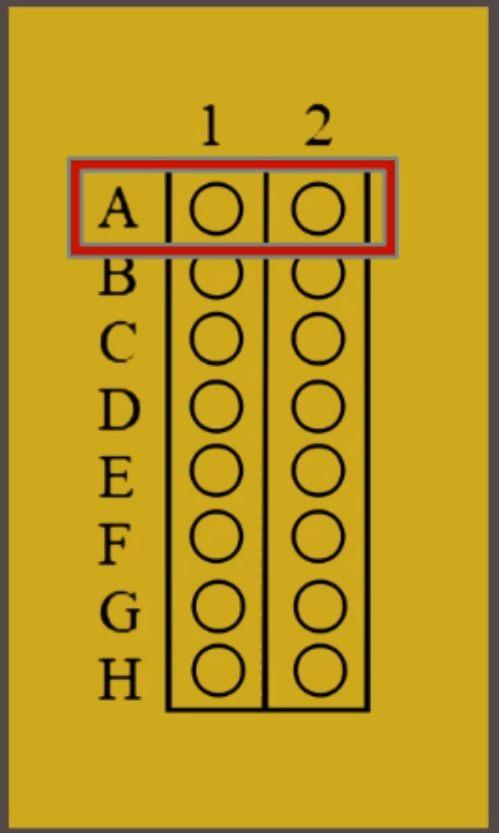
NB! These are concentrations in the well BEFORE CULTURE IS ADDED

÷ 2 ↓ $C = \frac{n}{V}$ ↑ x 2

CALCULATING MIC

Then we add 100 µl of stock culture to the wells.





$C_1V_1 = C_2V_2$

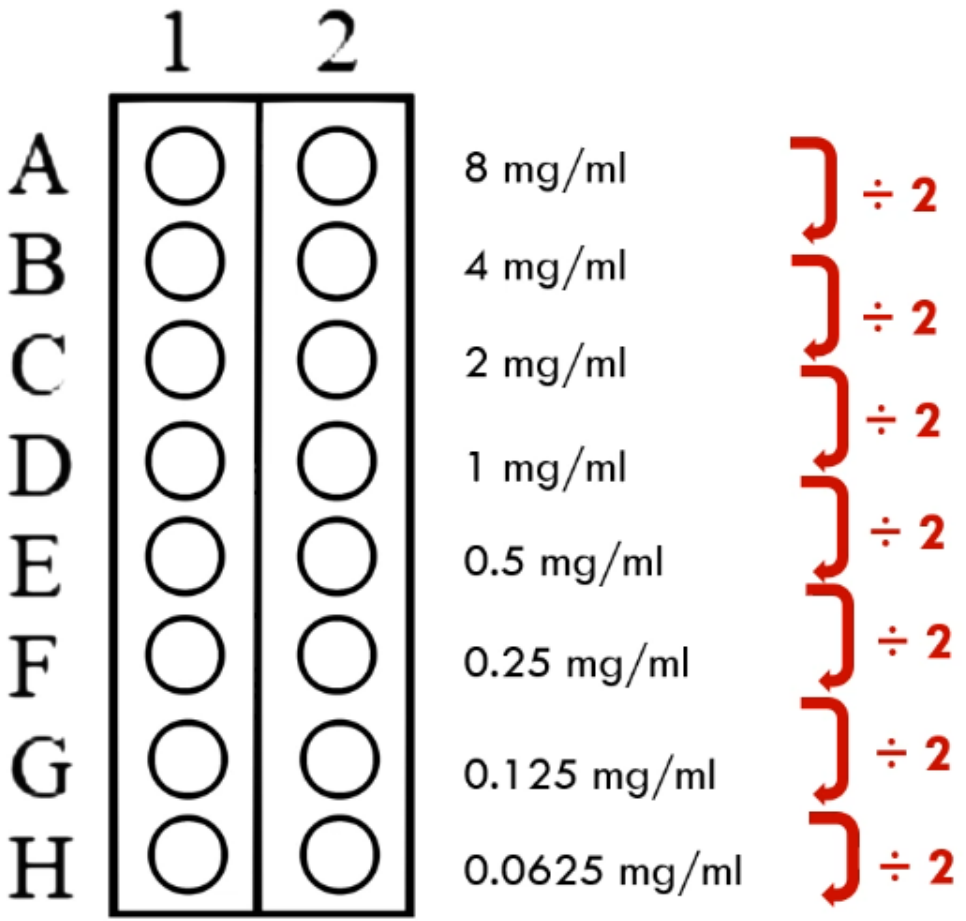
(16 mg/ml)(100 µl) = C_2 (200 µl)

C_2 = 8 mg/ml

OR

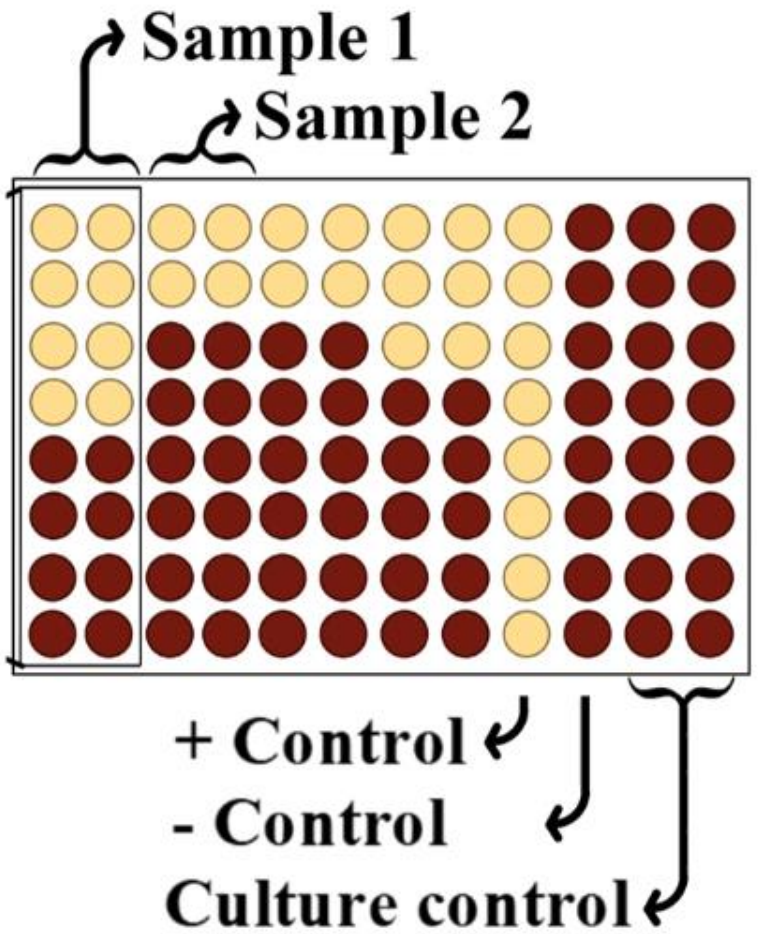
÷ 2 ↓ $C = \frac{n}{V}$ ↑ X 2

CALCULATING MIC



$$\div 2 \downarrow \quad C = \frac{n}{V} \uparrow \times 2$$

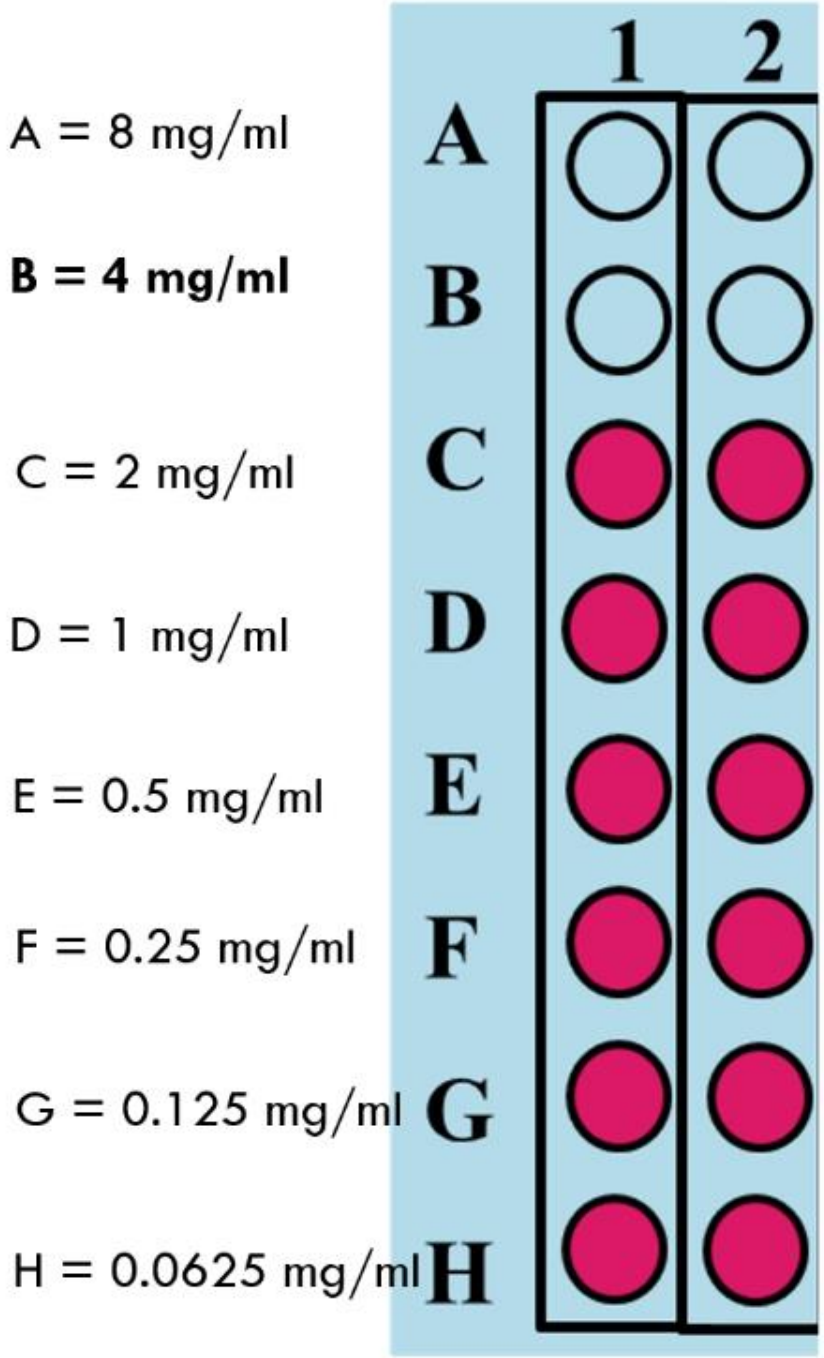
CALCULATING MIC



CALCULATING MIC

Example 1: Stock is 32 mg/ml

MIC = 4mg/ml



CALCULATING MIC

Example 2: Stock is 64 mg/ml

MIC = 0,25 mg/ml

A = 16 mg/ml

B = 8 mg/ml

C = 4 mg/ml

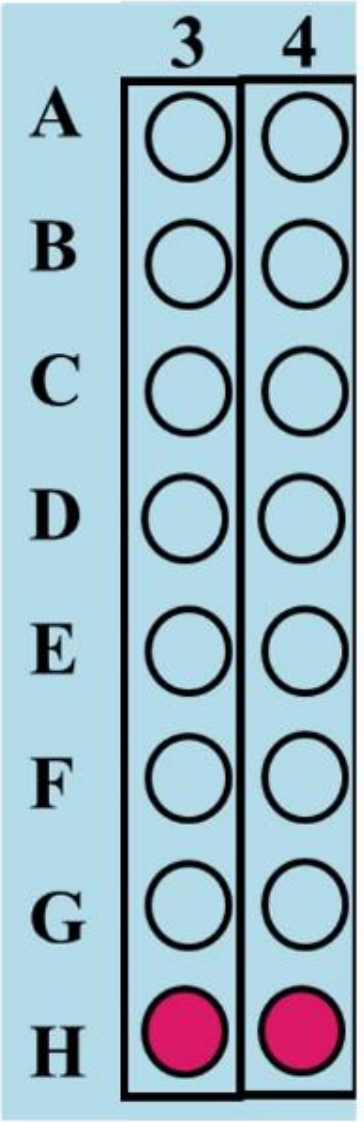
D = 2 mg/ml

E = 1 mg/ml

F = 0,5 mg/ml

G = 0,25 mg/ml

F = 0,125 mg/ml

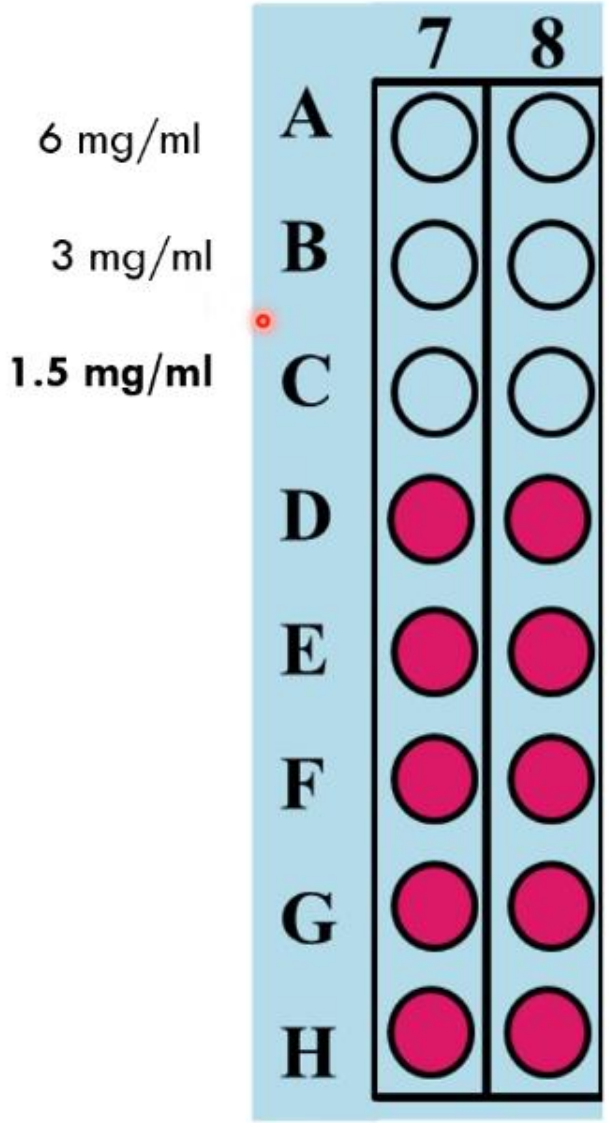


CALCULATING MIC

Example 3: Row B is 3 mg/ml

1. **What is the MIC value?**

MIC = 1.5 mg/ml



CALCULATING MIC

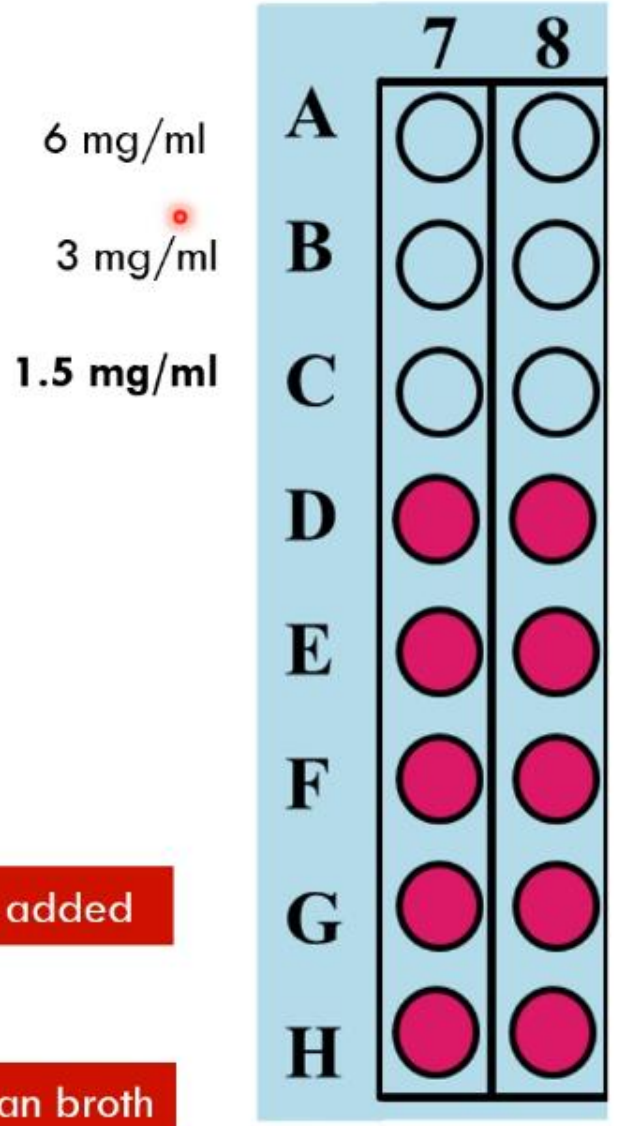
Example 3: Row B is 3 mg/ml

2. What is the stock concentration?

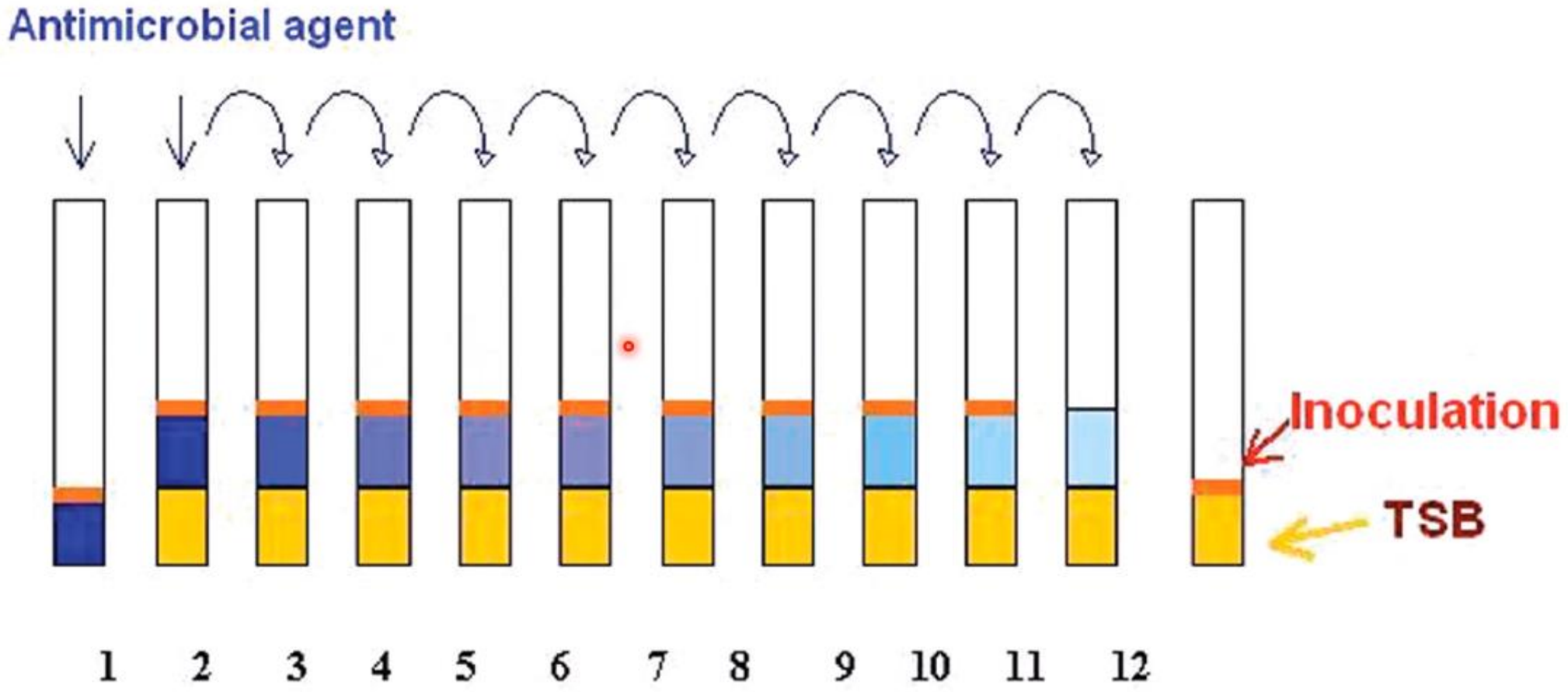
Row A = 6 mg/ml

6 mg/ml x 2 = 12 mg/ml — Conc. before culture was added

12 mg/ml x 2 = **24 mg/ml** — Stock conc. Added to clean broth



MACRO-DILUTION ASSAY (DEMO)



PRACTICAL 5

Tertiary area

- Performance assessment sheet
- Remove access clothing
- Tie hair up, remove jewelry, makeup
- Wash up (tip to elbow)
- Maintain non-touch technique

Secondary area

- Gown up (Kit 1)
- Cover ears
- Tie bows not knots
- Maintain non-touch technique

Primary area

- Start process
  - Spray operators hands with alcohol
  - Swab LFU
  - Open settle plates
  - Place white tile at the centre of the LFU
- Know which kit you need for your exercise
- Maintain non-touch technique
- End process
  - Close settle plates
  - Clear LFU
  - Swab LFU correctly

Incubation

- Products
- Settle plates
  - 37 °C for 24 hours
  - 25 °C for 7 days

Labeling

- Kit number
- Name
- Date
- Group

PRACTICAL 7

Introduction

- Sterilization
  - the process of making a product pathogen free
- Sterility testing
  - a procedure carries out in order to detect viable pathogens present in a preparation

Methods of sterility testing

Sterility of two of the preparations made in Practical 6 will be checked in this practical:

Soft lens solution
Eye ointment

Method 1: Membrane filtration

For aqueous, alcoholic or oily solutions

Method 2: Direct inoculation

Mostly for solid or semi-solid substances

Creams or ointments

Exercise 1

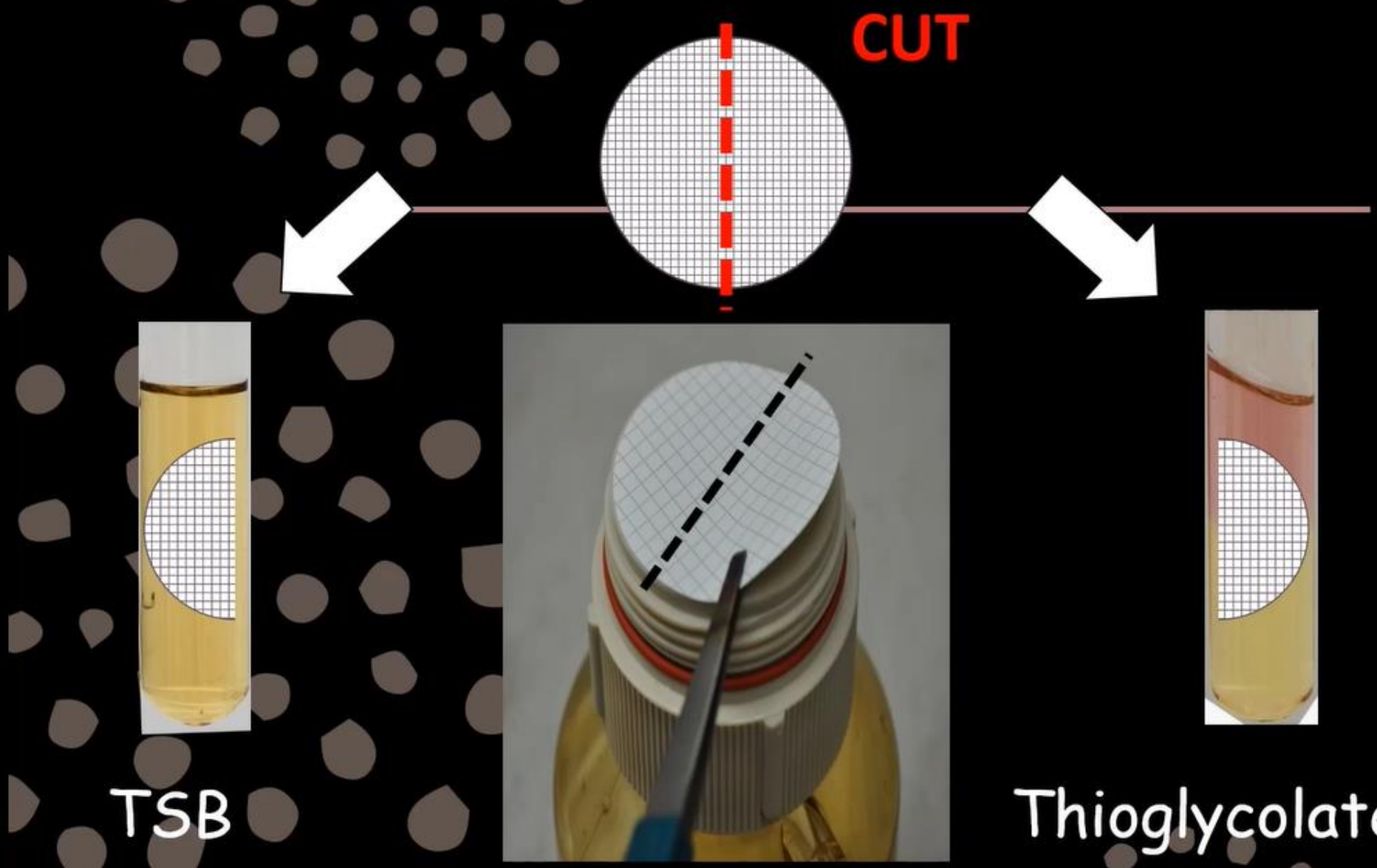
Sterility testing of soft lens solution using unit filter

(Millipore filter)

Steps:

- Assemble millipore filter as before, with membrane included
- Wet membrane with 10 ml sterile water
- Filter solution
- Wash membrane with 30 ml sterile water
- Aseptically cut membrane in half
- Place one half in aerobic broth test tube
- Place the other in an anaerobic broth test tube

Indirect inoculation



Exercise 2

Sterility testing of eye ointment using direct inoculation

Steps:

- Transfer +/- 1 g (large blob) of ointment into 10 ml of aerobic and anaerobic broth using a glass rod

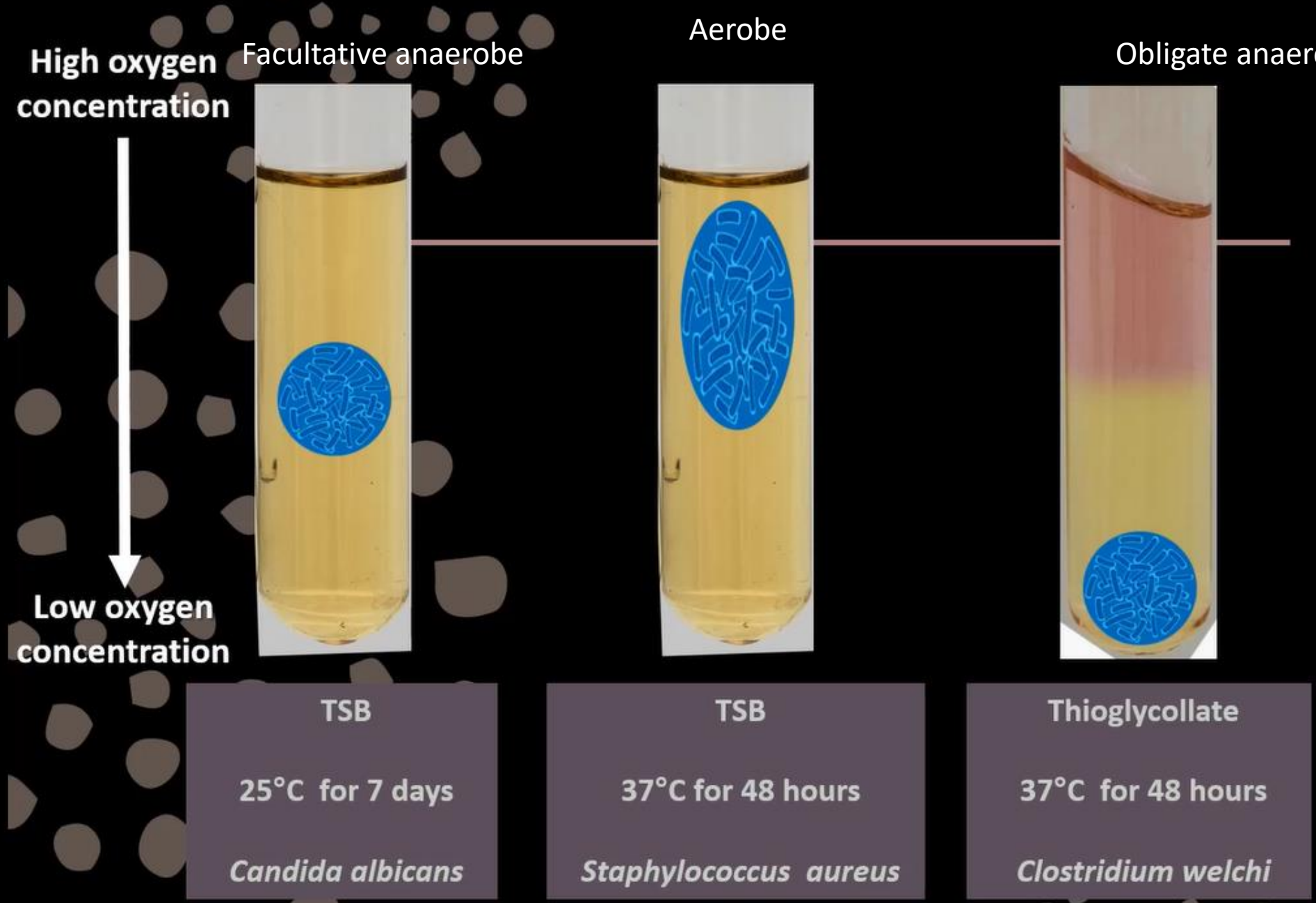
Direct inoculation

TSB
25 °C for 14 days

Thioglycolate
37 °C for 14 days

Important points

- Aerobic broth and anaerobic broth
- Try get some ointment in broth and not all on side of test tube
- Submerge membranes completely but without touching forceps to broth
- Incubation conditions vary:
  - TSB = 25 °C for 14 days
  - Thioglycollate = 37 °C for 14 days


High oxygen concentration
Low oxygen concentration
Facultative anaerobe
Aerobe
Obligate anaerobe
TSB
25°C for 7 days
Candida albicans
TSB
37°C for 48 hours
Staphylococcus aureus
Thioglycollate
37°C for 48 hours
Clostridium welchi

Controls

Positive controls

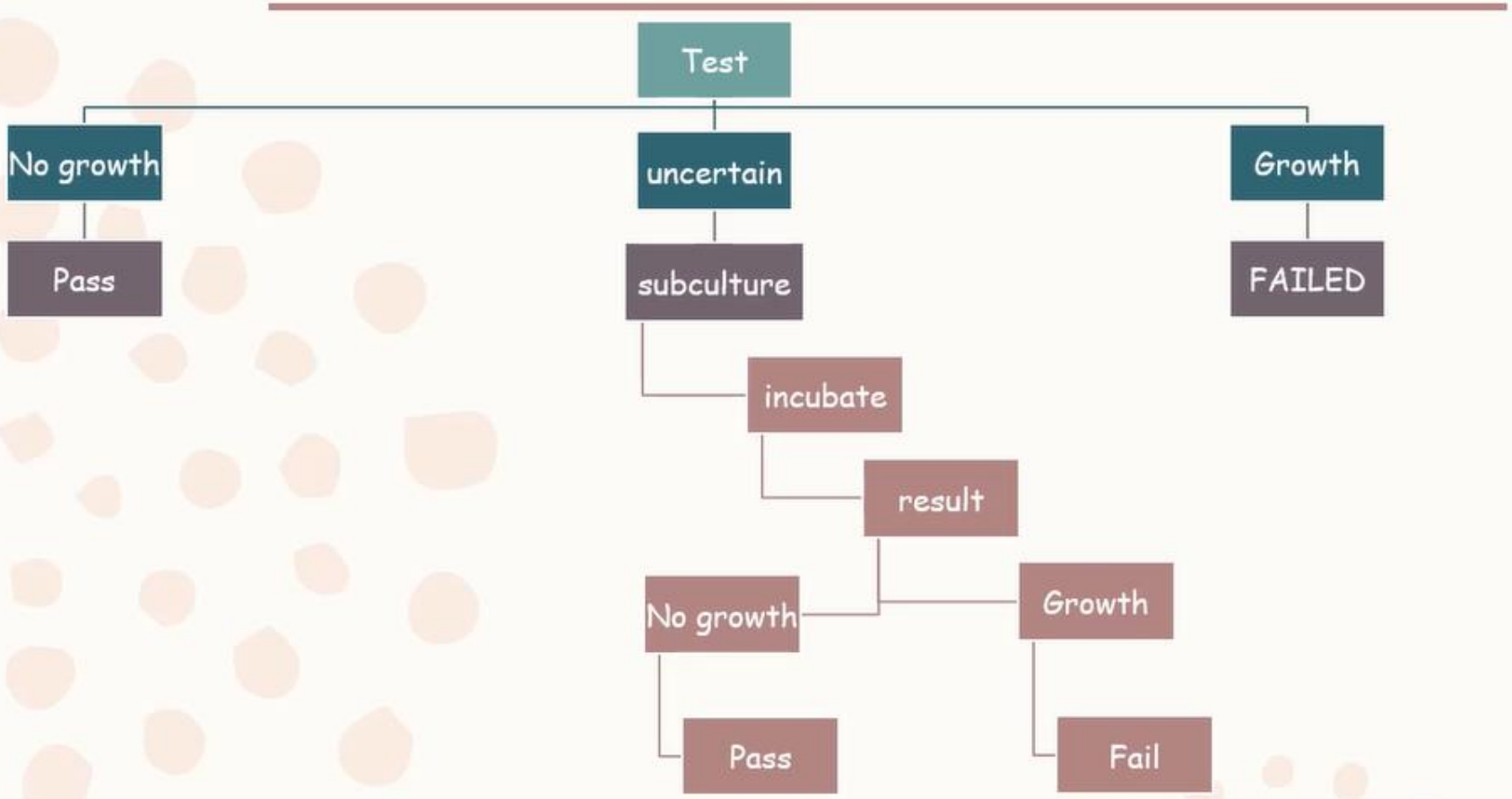
- Media inoculated with micro-organisms
- Test if media can support growth
- To see what contamination would look like

Negative controls

- Media with nothing added
- Test if media itself is sterile
- See what media looks like without contamination

Type of control (negative/ positive)	Media	Incubation conditions	Purpose of control	Inoculated with/ not inoculated
Negative	Tryptone Soya broth	25 °C for 48 hours	Ensure broth sterility	X
Negative	Thioglycolate broth	37 °C for 24 hours	Ensure broth sterility	X
Positive	Tryptone Soya broth	37 °C for 24 hrs	Suitability of media for aerobic growth and see what contamination looks like	*S. aureus*
Positive	Thioglycolate broth	37 °C for 24 hrs	Suitability of media for anaerobic growth and see what contamination looks like	*C. welchi*
Positive	Tryptone Soya broth	25 °C for 48 hrs	Suitability of media for yeast growth and see what contamination looks like	*C. albicans*

Interpretation of results



REVISION

EXAMPLE OF LABELLING

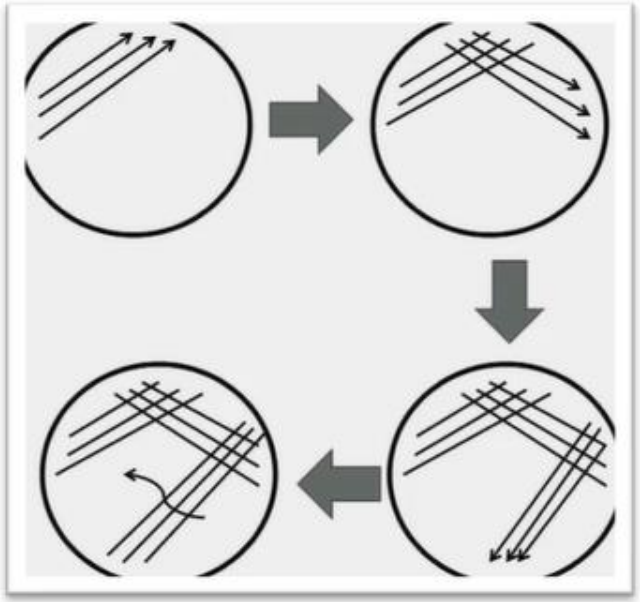
Name
Date
Group
Practical
Technique/procedure (E.g. Streak plate)
Micro-organism used
Incubation conditions

YES

???
Date
Practical

NO

STREAK PLATE



Parallel lines
Do not dip into the agar
FLAME LOOP between a set of lines drawn.

What is the purpose of a streak plate?

To ensure purity of culture and attain single colonies

04:14

SPREAD PLATE

- Do not touch the hook
- Spread as evenly as possible without touching the walls of the petri dish.
- Make sure to keep the spreader flat on the agar surface

What is the purpose of a spread plate?

To observe uniform growth of culture.

DISC DIFFUSION / WELL DIFFUSION

- Spacing of disc or wells
- Know which antimicrobial to use against which pathogen (don't only study one set of antimicrobial per pathogen)

COMMERCIAL PRODUCTS

Product	Concentration	Agar diffusion (mm)		
		E.coli	S.aureus	C.albicans
Orochlor	20%	1	7	0
Savlon	0.3%	8.8	14.25	16
Domestos	5%	5	6	2
Dettol	4.8%	3	12.67	10
Listerine	0.5%	0	0	1.67
Andolex	9%	6.33	7.5	5.5

CULTURE CONDITIONS

Anaerobe

*Clostridium welchi***, *Streptococcus mutans*

Aerobe

Mycobacterium tuberculosis, Staphylococcus aureus.

Gram positive bacteria

Staphylococcus aureus.

Gram negative bacteria

Escherichia coli, Pseudomonas aeruginosa

Yeast

Candida albicans

Mould

Aspergillus niger

Fastidious Microorganism

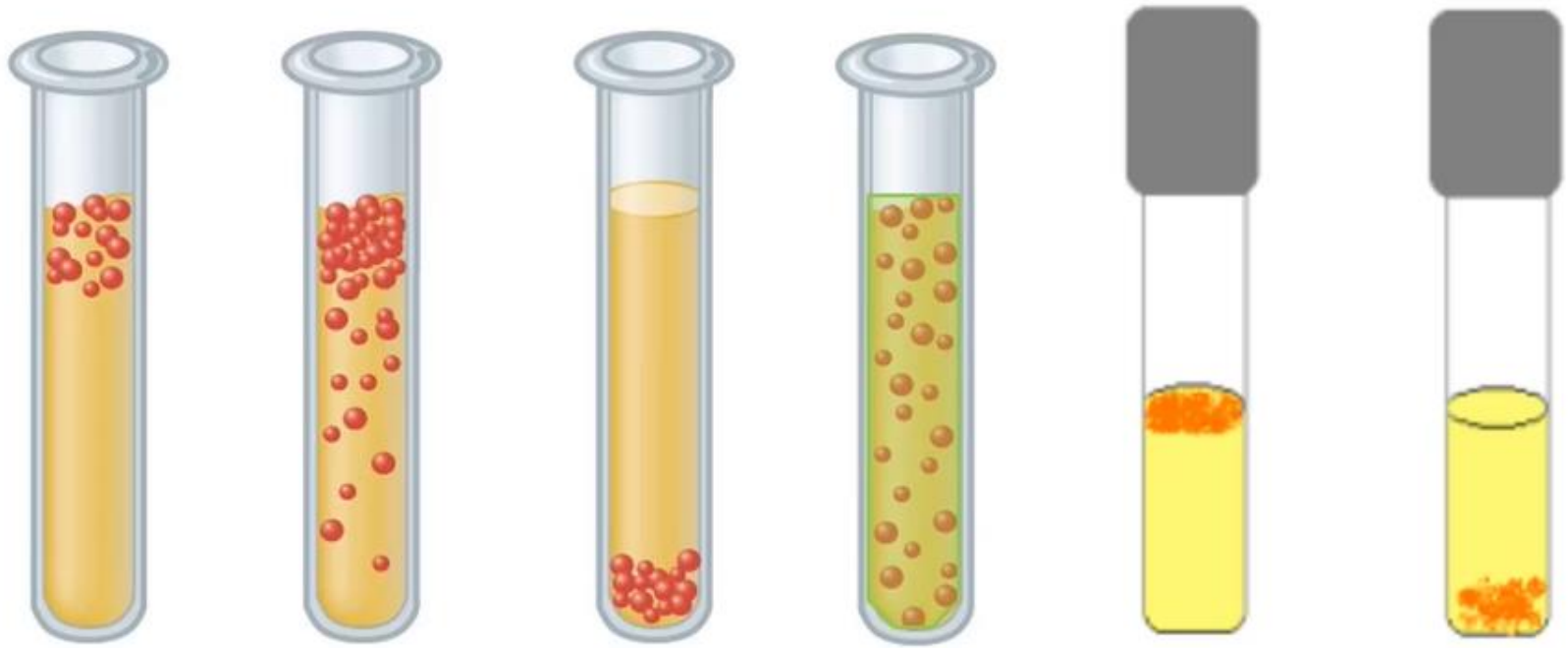
Neisseria gonorrhea, Streptococcus mutans

37 °C
24 hours

25 °C
48 hours/
7 days

GROWTH PATTERNS

Examples of each type



Aerobe

Facultative anaerobe

Obligate anaerobe

Pigment

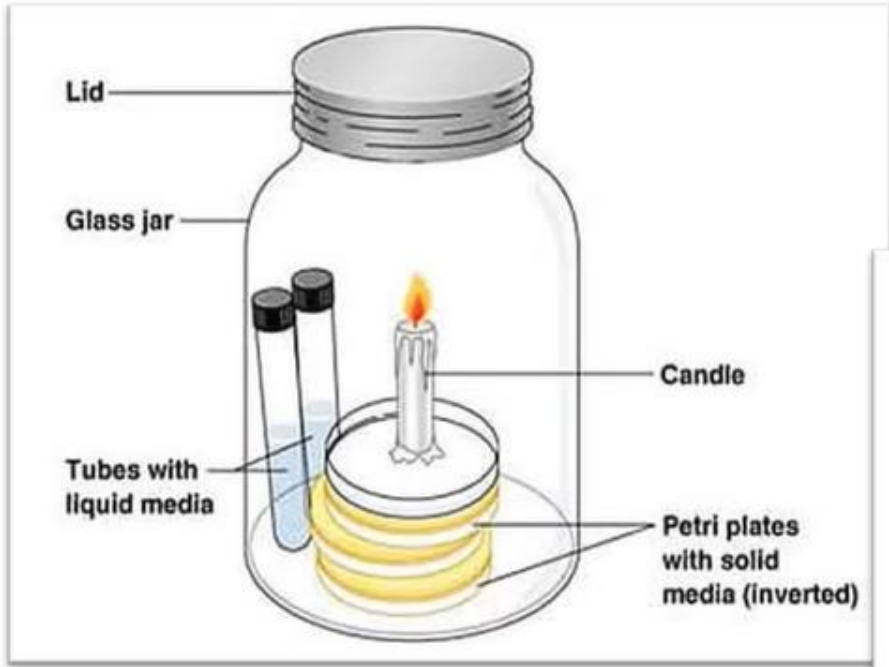
Pellicle

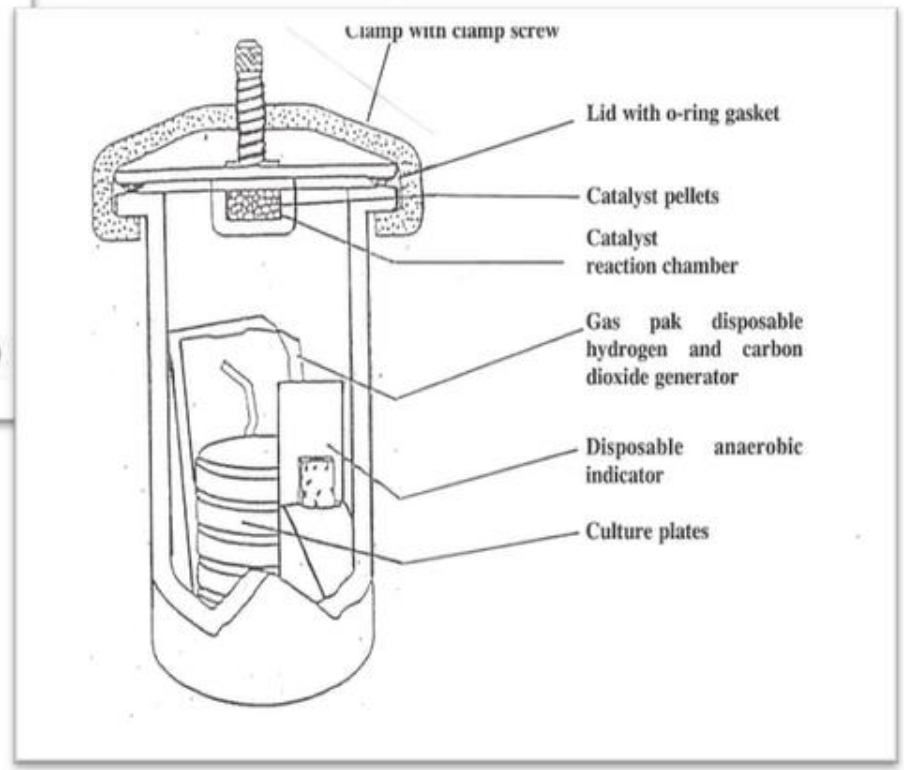
Sediment

GROWTH MEDIA

Medium	Pathogen	Example
Nutrient agar (NA) or broth (NB)	Bacteria	*S. aureus, P. aeruginosa*
Tryptone Soya agar (TSA) or broth (TSB)	Yeast, bacteria	*C. albicans, S. aureus*
Thioglycollate broth (TGB)	Anaerobes	*C. welchi, P. acnes*
*Sabouraud's dextrose broth (SDB) or agar (SDA)	Yeast and dermatophytes	*C. albicans, Trichophyton spp., Microsporum ssp.*
*Mueller Hinton broth supplemented with 5% sheep's blood (MHB) or agar (MHA)	Coagulase bacteria	*Streptococci (mutans, pyogenes)*
Rogosa broth (RB) or agar (RA)	Acid environment	*Lactobacillus*

ANAEROBIC MICROORGANISM





AIR SAMPLING

Understand
Active & Passive
forms.

Reuter
Centrifugal
Sampler

Running time: 640 seconds
Volume of air : 288 L
Size detection limit : 2 µm

Running time: 4 minutes (240 seconds)
Volume of air : 280 L
Size detection limit: 0.4 µm

Sieve
Air
Sampler

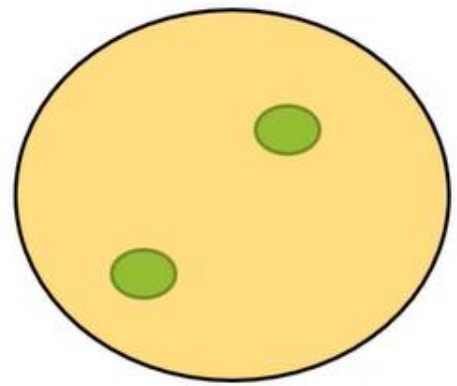
Air Sampling Calculation

CFU/L= (No. of colonies/ rate of V of air)

2 colonies; SAS air sampler

CFU/L (sec) = 2/(288/640)

SAS = 4,44 CFU/L per second



PRACTICAL DEMO RESULTS

Area tested	No. of people in area	Air sampling results			
4th floor toilets	7	No. of colonies on SAS plate (bacterial)	225	Bacterial CFU/l (SAS)	500156,25
		No. of colonies on SAS plate (fungal)	15	Fungal CFU/l (SAS)	33343,75
		No. of colonies on RCS strip (bacterial)	351	Bacterial CFU/l (RCS)	300857,14
		No. of colonies on RCS strip (fungal)		CFU/l (RCS)	n/a
3rd floor ladies bathroom	7	No. of colonies on SAS plate (bacterial)		(SAS)	171164,58
		No. of colonies on SAS plate		SAS)	124483,33
		No. of colonies on RCS strip (bacterial)		CFU/l (RCS)	96857,14
		No. of colonies on RCS strip (fungal)	11	Fungal CFU/l (RCS)	9428,57
Area tested	No. of people in area	Air sampling results			
Entrance to hospital (3rd floor)	15	No. of colonies on SAS plate (bacterial)	73	Bacterial CFU/l (SAS)	162272,92
		No. of colonies on SAS plate (fungal)	8	Fungal CFU/l (SAS)	17783,33
		No. of colonies on RCS strip (bacterial)	44	Bacterial CFU/l (RCS)	37714,29
		No. of colonies on RCS strip (fungal)	34	Fungal CFU/l (RCS)	29142,86
3rd floor small CNS lab	18	No. of colonies on SAS plate (bacterial)	4	Bacterial CFU/l (SAS)	8891,67
		No. of colonies on SAS plate (fungal)	17	Fungal CFU/l (SAS)	37789,58
		No. of colonies on RCS strip (bacterial)	4	Bacterial CFU/l (RCS)	3428,57
		No. of colonies on RCS strip (fungal)	17	Fungal CFU/l (RCS)	14571,43

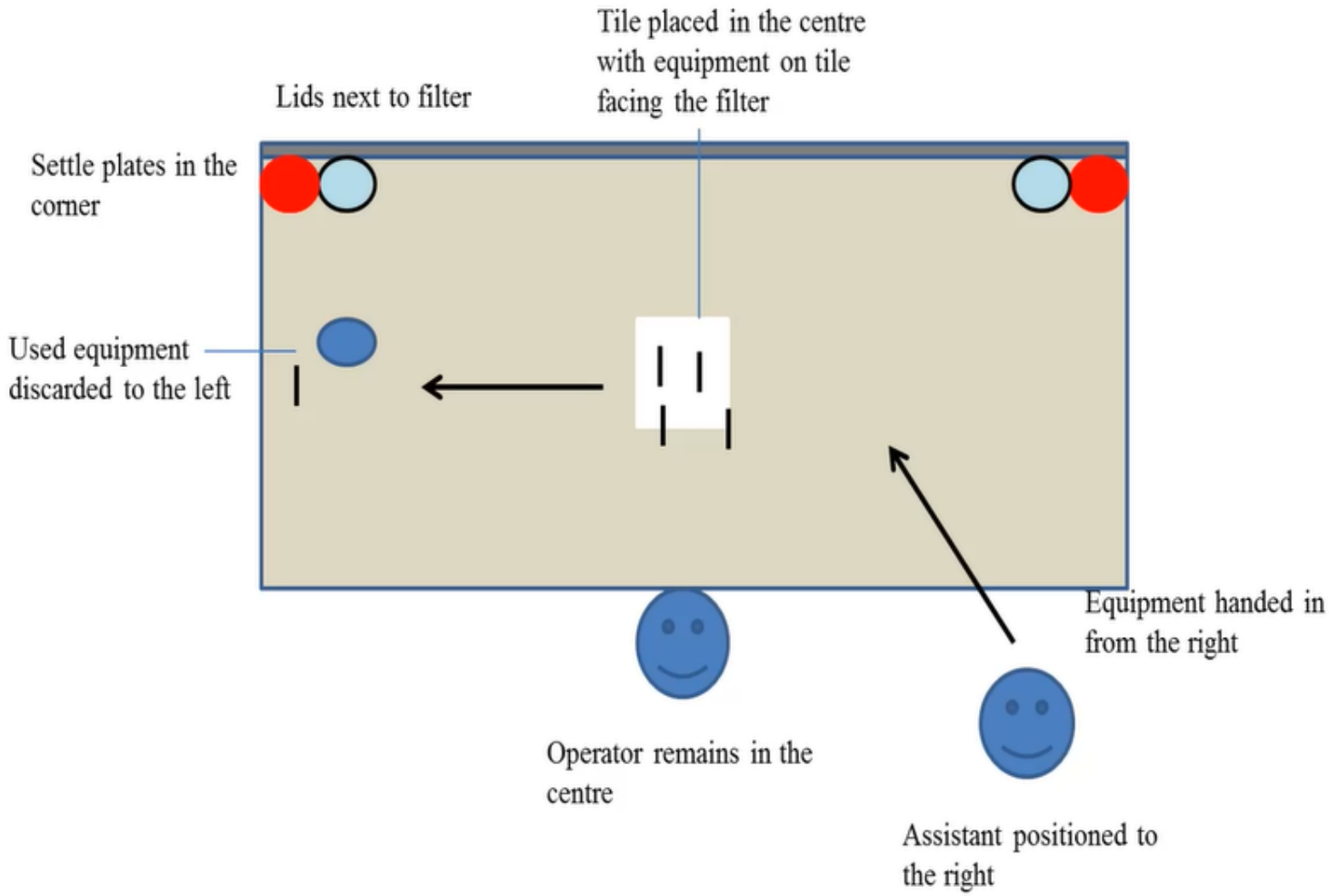
$$\left(225 \Big/ \frac{288}{640,2}\right) x\ 1000$$

ASEPTIC TECHNIQUE

Classes of products that are not suitable for sterilization via heat treatment require aseptic processing.

Examples?

ASEPTIC TECHNIQUE


Tile placed in the centre with equipment on tile facing the filter
Lids next to filter
Settle plates in the corner
Used equipment discarded to the left
Equipment handed in from the right
Operator remains in the centre
Assistant positioned to the right

STERILITY TESTING

Difference in incubation periods for each…

Controls

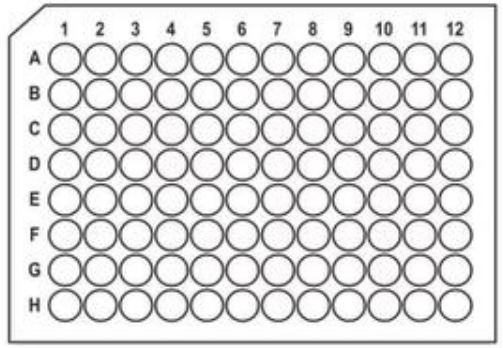
Positive controls

- Sterile media inoculated with micro-organisms
- Test if **media can support growth**
- To see what contamination would look like

Negative controls

- Sterile media with nothing added
- Test if media itself is **sterile**
- See what media looks like without contamination

CALCULATING MIC



Stock concentration : 32 mg/ml

1. **First, we added 100 µl of broth to each well**
2. **Then we added 100 µl of sample to the first row**
3. **Then we serially diluted the samples down**

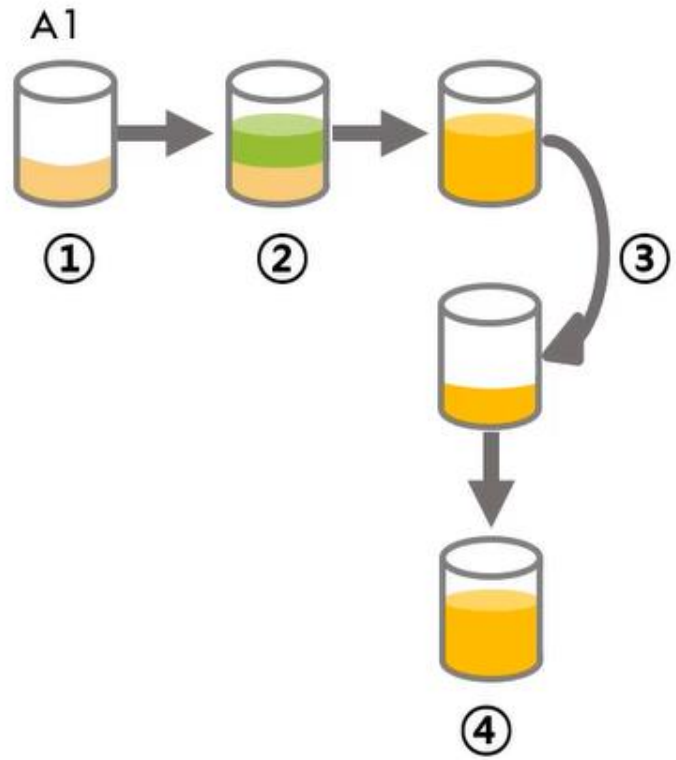
Therefore the concentration halved

4. Then we added 100 µl of the stock culture.

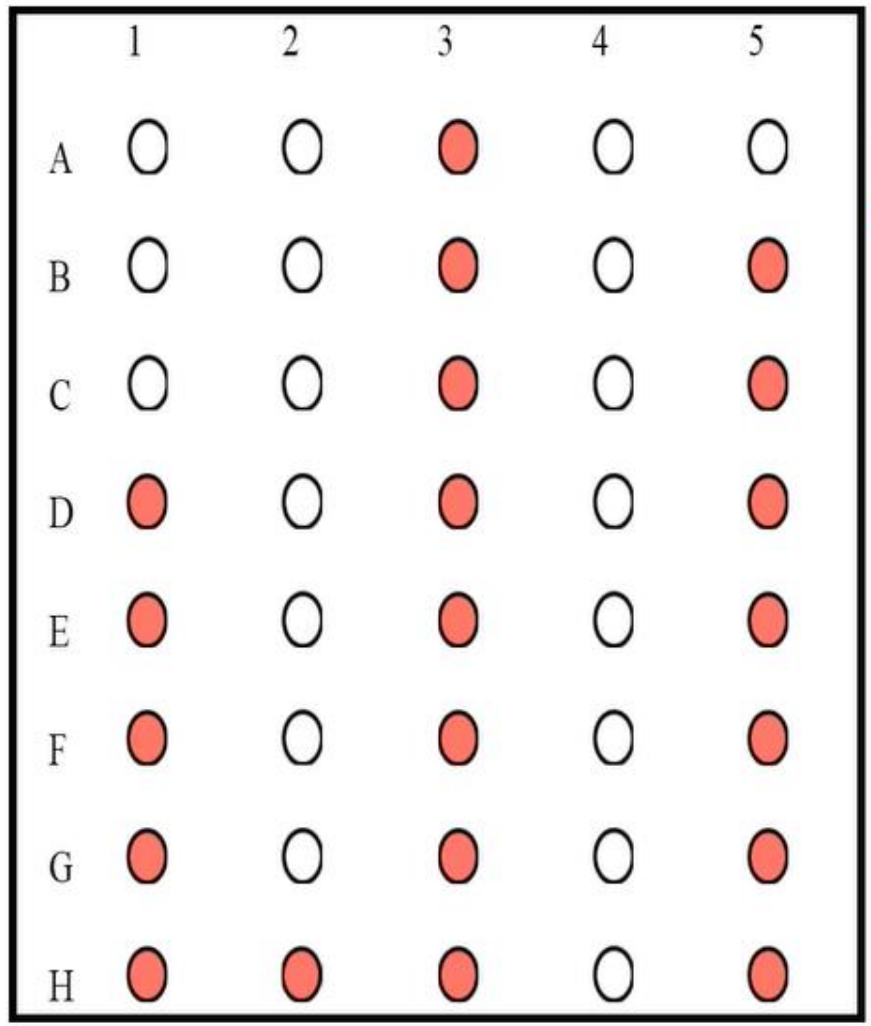
Therefore the concentration halved again

What is the concentration in Row A?

8 mg/ml



Interpreting MIC results



Starting (stock) concentration: **32mg/ml**

What is the concentration in well A?

8 mg/ml

1= 2 mg/ml

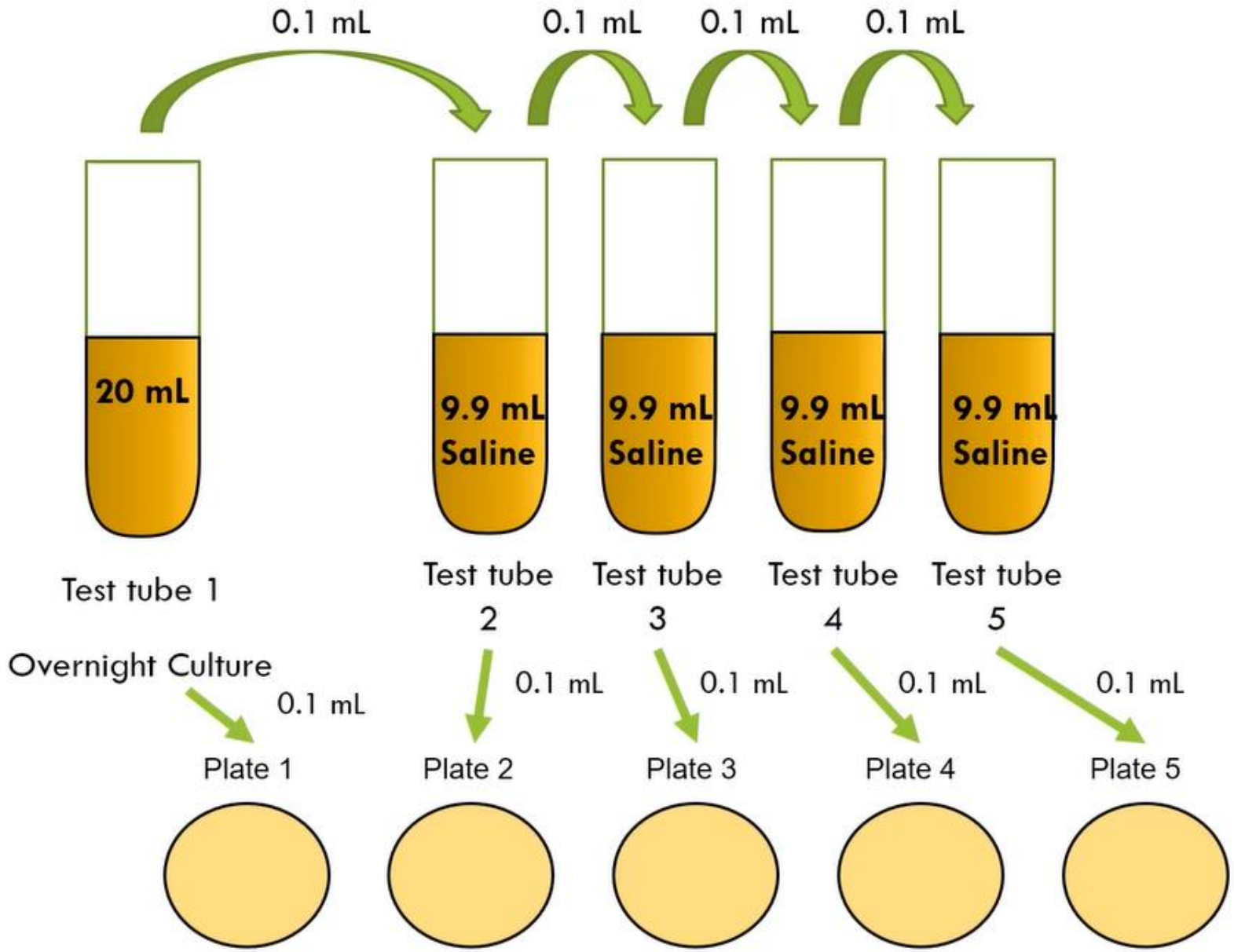
2= 0.125 mg/ml

3= >8 mg/ml Negative control

4= ≤ 0.0625 mg/ml Positive control

5= 8 mg/ml

PRESERVATIVE EFFICACY



PRESERVATIVE EFFICACY

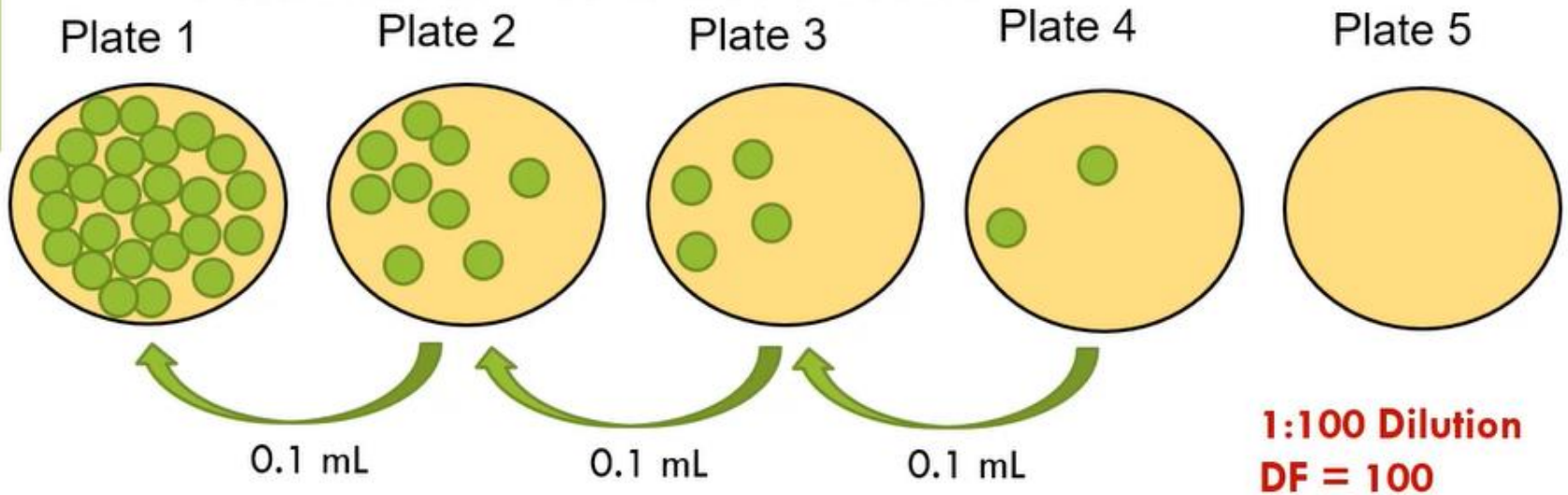


Plate 4
2 CFU in 0.1 mL

$$\frac{2\ CFU}{0.1\ mL} = \frac{x}{1\ mL} \qquad x = 20\,\frac{CFU}{mL}$$

20 CFU X 100 X 100 X 100 X 20

= 400 000 000 CFU in 20 ml

= 400 000 000 CFU in 20 ml **/ 20**

= 20 000 000 CFU in 1 ml